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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VI. ON THE VIRULENCE OF THE OVERGROWTH IN THE LYSSED CULTURES OF *BACILLUS PESTIS CAVIÆ* (M. T. II).

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When susceptible bacteria are grown on broth in the presence of a suitable lytic agent, the initial turbidity of the culture gradually disappears because of the lysis of the bacteria. In exceptional cases the lysis may be complete and permanent, resulting in sterilization of the culture. But more often a few bacteria fail to undergo lysis and multiply in spite of the presence of lytic agent in the medium, thus giving rise to more or less profuse overgrowth of resistants, following a temporary clearing of the culture (1).

In the case of *B. coli*, Bordet and Ciuca (2) found that the overgrowth differs from the original culture, in addition to its resistance to lysis, by its active motility; and Gratia (3) found further that the overgrowth is more highly virulent. The heightened virulence of bacteria resistant to lysis was observed also by other investigators and has been suggested as the source of the failures of bacteriophage to influence favorably the course of infection in certain instances (4-7).

In view of the fact that the virulence of a strain M. T. II of *B. pestis caviæ* was carefully established during several years of study of experimental mouse typhoid at The Rockefeller Institute (8-10), we thought that a lysis-resistant overgrowth of this culture would offer exceptional material for comparison of its virulence with that of the original culture.

Isolation of Resistants.

In order to obtain pure strains of resistant bacteria, the stock culture M. T. II was grown in broth for 24 hours, in the presence of each of the two lytic filtrates

active against this organism. These lytic filtrates were selected from several isolated by us at different times from the stools of mice recovering from experimental mouse typhoid, because they were typical of the variations in the character of lytic activity observed in these filtrates.¹

The overgrowth which was present in the cultures after 24 hours of incubation at 37°C. was streaked out on agar plates. Single colonies appearing on the plates were repeatedly transferred to fresh agar (each time from a single colony) in order to eliminate all traces of lytic principle. After seven successive transfers, 24 hours apart, each of the cultures was returned to broth. They proved to be free from lytic principle when filtered and tested against the original susceptible stock culture of M. T. II. Broth cultures themselves were tested at the same time for their susceptibility to lysis by each of the lytic filtrates in turn. As the results recorded in Protocol I demonstrate, each culture obtained from the overgrowth was resistant to lysis by the lytic agent used in its production, but only one of the cultures was resistant also to the activity of the second lytic agent.

Protocol I.

Identification of Resistant Isolated from Lysed M. T. II Culture.

Lytic agents used to obtain resistant	Character of growth in broth	Agglutinability with stock M. T. II serum 1:2000	Resistance or susceptibility to lysis	
			Resistant W-Little R ₇ *	Resistant W-178 R ₇ *
W-Little.....	Agglutinated	Positive	Resistant	Resistant
W-178.....	Diffuse	Positive	Susceptible	Resistant

* The legend R₇ added to the name of each culture indicates that it represents a resistant (R) variant of the seventh (7) passage on agar.

The identity of these cultures with the parent culture of M. T. II was checked by the agglutination test, in which a stock anti-M. T. II serum diluted 1:2000 was used as agglutinin.

It was observed that in some cases the overgrowth appeared diffuse; in others it was spontaneously agglutinated. Since this difference in the appearance of overgrowth has been stressed in the literature (6, 7) we have carried out all the experiments with Strains W-178 and

¹ We are greatly indebted to Dr. Ida Pritchett for placing at our disposal the greater part of the animals used for isolation of lytic agents. In passing, it should be recorded that the lytic filtrates yielded by the examination of over 300 mice were, for the most part, active only against *B. dysenterizæ*. Next in frequency were those active against *B. enteritidis* Gaertner, and least frequently those active against the infecting organism M. T. II.

W-Little as representing this difference in growth. In order to avoid spontaneous return of susceptibility to lysis, the cultures were grown on agar and transplanted only as often as the experiments required. Whenever resistant bacteria were needed for the test, a subculture from agar was made into broth and used as such after 18 hours of incubation at 37°C.

Virulence of the Resistants by Feeding.

After having carried the resistant strains W-Little R₇ and W-178 R₇ for four more generations on agar (eleven in all) we attempted to determine their virulence as compared with that of the original strain of *B. pestis caviæ*.

Protocol II.

Virulence of Resistants by Feeding.

Bacteria fed		Group A (control) Stock M. T. II	Group B W-Little R ₁₁	Group C W-178 R ₁₁
No. of bacteria given		3,000,000	3,000,00	3,000,000
No. of mice surviving by days	5th day	25	25	25
	6th "	23	25	25
	7th "	19	25	25
	8th "	15	25	25
	9th "	12	25	25
	10th "	10	25	25
	11th "	9	25	25
	12th "	8	25	25
	13th "	8	25	25
	14th "	8	25	25

The animals used for these experiments came from special stock kept at The Rockefeller Institute, and known to have been free from spontaneous mouse typhoid for a period of years. It was hoped that these animals would show no heightened resistance to the infection, and would present an ideal object for the study to be undertaken.

75 mice of approximately 25 gm. weight each were divided into three equal groups. To each mouse of the first group (Protocol II, A) was given, by means of a stomach tube, 0.5 cc. of an 18 hour broth culture of *B. pestis caviæ* (M. T. II), diluted to contain about 3,000,000 bacteria in each dose. The mice of the second group (Protocol II, B) each received about 3,000,000 bacteria (in 0.5 cc. volume)

from an 18 hour broth subculture from the eleventh generation of a resistant W-Little R₁₁ on agar. The mice of the third group (Protocol II, C) each received 0.5 cc. of broth containing about 3,000,000 bacteria of an 18 hour broth subculture of W-178 R₁₁. Each animal was placed in an individual jar, its ordinary ration was supplied daily, and the litter was changed three times per week. No symptoms were noted until the 4th or 5th day after the feeding of bacteria, when certain animals in Group A appeared ill, and from the 6th day deaths began to occur, as indicated on Protocol II. The experiment was interrupted on the 15th day, after no deaths had occurred among the animals for 3 days. At this time all the animals receiving resistant bacteria (Groups B and C) were living, whereas only eight animals out of twenty-five (about 32 per cent) survived the feeding of the original M. T. II.

*Susceptibility to Infection by B. pestis caviæ (M. T. II) of Mice
Surviving the Feeding of Resistants.*

In the next two experiments the resistants of the thirteenth and fourteenth generations on agar were used. The method was the same as in the first experiment and the results may be summarized by stating that the mortality of controls (corresponding to Group A on Protocol II) was 62 and 66 per cent respectively, whereas all the animals but two of those fed the resistants remained alive. That these two deaths did not arise from mouse typhoid infection is rendered probable by the fact that at the autopsy the blood and internal organs were found to be free from *B. pestis caviæ*.

Since the failure of resistants to infect mice was contrary to what was expected on the basis of earlier reports in the literature (1-5, 11), it was thought advisable to ascertain the susceptibility of the surviving mice in the above experiments to infection with the parent culture M. T. II; if these animals prove susceptible to subsequent infection with M. T. II, their earlier resistance to W-Little R₁₄ and W-178 R₁₄ respectively can be ascribed to a change in virulence in these cultures.

Accordingly, twenty mice each of those (twenty-five) surviving feeding with W-Little R₁₄ and W-178 R₁₄ respectively (corresponding to Groups B and C on Protocol II) were divided into two groups. The first subgroup of ten mice from each group received a suspension of an 18 hour broth culture of virulent M. T. II by mouth, the other by intraperitoneal injection. Similarly, half of twenty normal mice were given the same bacterial suspension by mouth, and the other half by intraperitoneal injection. The animals were cared for exactly as in the earlier ex-

periments, except that those injected intraperitoneally were placed in larger containers—five mice in each. Protocol III illustrates the results obtained.

The remaining eight mice surviving the feeding of resistants² were killed and examined for signs of invasion by the bacteria which had been fed to them. As in the case of two mice of this series which died

Protocol III.

Susceptibility of Surviving Mice to Infection by M. T. II.

		Mice surviving the feeding of resistants 15 days previously				Normal controls	
		Survivals of Group B (fed W-Little R ₁₄)		Survivals of Group C (fed W-178 R ₁₄)			
		10	10	10	10	10	10
No. of mice							
Mode of infection		By mouth	Intraperi- toneal	By mouth	Intraperi- toneal	By mouth	Intraperi- toneal
No. of virulent bacteria given (about)		5,000,000	2,000,000	5,000,000	2,000,000	5,000,000	2,000,000
No. of deaths per day	1st day		3		4		3
	2nd "		3		3		6
	3rd "		1		3		1
	4th "		2				
	5th "		1			1	
	6th "			1		2	
	7th "	2		3			
	8th "	1				1	
	9th "	3		1		2	
	10th "						
	11th "	1					
	12th "			1		1	
	13th "						
	14th "						
No. of survivals.....		3	0	4	0	3	0

earlier, the internal organs of these mice were sterile, but the intestinal contents showed the presence of bacteria resembling the parent strain M. T. II in their lack of ability to ferment lactose, in the production of H₂S, and in their immunologic properties. They differed

² Twenty mice of each series of twenty-five were used in the preceding experiment, and one animal in each group died from intercurrent cause, thus leaving four mice in each group (see experiment Protocol II).

only in their power to resist lysis by homologous lytic agents, thus establishing their identity with the material fed to the mice. In other experiments of similar nature, the intestinal contents yielded an occasional colony susceptible to lysis; the bulk of bacteria fed to the mice seemed, however, to have remained in the intestinal contents without becoming susceptible to lysis, and without entering into the blood for at least 14 days. If this be proven to be the case with larger experimental material, it would suggest the loss of invasive power by the resistants.

Virulence of Resistants by Intraperitoneal Injection.

The fact that mice surviving the feeding of resistants were still susceptible to subsequent infection by the virulent parent M. T. II strain, together with the finding that the resistants lack invasive power, would indicate that their failure to kill animals in earlier experiments was due to this change in virulence, as suspected. However, we thought it of interest to inquire whether this change in virulence was limited to their loss of invasive power only, or to some more radical change. For this reason we compared their power to infect mice by parenteral route with that of the parent strain M. T. II. Accordingly, three series of fifteen mice each were given, by intraperitoneal injection, varying doses of the original M. T. II, W-Little R₁₄, and W-178 R₁₄ respectively, as indicated on Protocol IV, and the time of death of each mouse was noted. It will be seen from this protocol that whereas all the mice receiving the original M. T. II injection were dead before the expiration of 5 days (after injection), only three mice out of a total of thirty animals injected with resistants were dead up to the 10th day, when the experiment was terminated. We are inclined to attribute the death of these three mice to other causes rather than to the infection, particularly since in two subsequent experiments analogous to the one just described—except that the dose of bacteria was doubled and trebled respectively—only one mouse died, out of a total of 60 receiving resistant bacteria intraperitoneally, whereas the mortality of mice receiving the original M. T. II culture was invariably 100 per cent. These experiments, in our opinion, indicate that at least in the case of *B. pestis caviæ* isolation of cultures resistant to lysis by bacteriophage results in obtaining an avirulent strain of this organism.

Protocol IV.
The Loss of Virulence by the Resistant Subcultures of M. T. II as Tested by Intraperitoneal Injection.

	Original M. T. II (susceptible to lysis)					W-Little R ₁₁ (resistant to lysis)					W-178 R ₁₁ (resistant to lysis)				
No. of bacteria given intraperitoneally*	777,000					1,900,000					7,000,000				
No. of mice injected.....	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
No. of mice surviving after 10 days..	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
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	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
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	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
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	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
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	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
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	777,000					1,900,000					7,000,000				
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	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	None					None					None				
	777,000					1,900,000					7,000,000				
	1	2	3	4	5	1	2	3							

Protocol V.
Virulence of the Reverted W-Little R₁₁ by Intraperitoneal Injection.

	A			B			C		
	Original culture of M. T. II			Grown for 18 hrs. on broth			W-Little R ₁₁ (resistant variant)		
Character of growth on broth.....	Diffuse			Agglutinated			After 20 daily passages on broth		
Susceptibility to lysis by lytic agent W-Little	Susceptible			Not susceptible			Diffuse		
No. of bacteria given intraperitoneally*	966,000	2,900,000	8,700,000	1,366,000	4,100,000	12,300,000	967,000	3,100,000	9,700,000
No. of mice injected	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
No. of mice surviving after 10 days (virulence).....	None	None	None	All	All	Four	None	None	None
Time of death after injection in days	10								
	9								
	8								
	7								
	6								
	5								
	4								
	3								
	2								
	1								
	0								

* These counts were obtained by plating samples of suspensions of bacteria immediately after injection.

Virulence of W-Little R₁₄ after Its Recovery of Susceptibility to Lysis.

It is well known that bacteria isolated from the overgrowth of lysed cultures usually revert to the original type and become susceptible to lysis after a more or less prolonged cultivation on media free from lytic agent. Since the preceding experiments indicated that there may exist an interdependence between the lack of susceptibility to lysis and the loss of virulence in the culture of *B. pestis caviae*, it seemed desirable to find out whether the return of susceptibility to lysis will be followed by a return of virulence in such cultures.

For this purpose the fourteenth generation of a resistant strain (W-Little R₁₄) was subcultured from agar into broth, and from the latter daily transfers to broth were continued for 20 days, after which the culture thus obtained was tested for its susceptibility to lysis, as well as for its virulence to mice, by the intraperitoneal route. For the sake of comparison parallel tests were made with the culture of the original M. T. II, as well as with an 18 hour broth culture obtained by inoculating into sterile broth a small loop from the old agar slant W-Little R₁₄, which presumably retained its resistance to lysis by the phage W-Little. The procedure followed in this experiment was entirely analogous to that of the preceding experiment (Protocol IV), except that in addition to other tests, each culture was subjected to a control test of its susceptibility to lysis. The results of the virulence test are given in Protocol V. Similarly, the twentieth passage in broth from W-Little R₁₄ proved capable of killing mice when given by mouth (Protocol VI).

It is evident, then, that when full reversion to susceptibility to lysis, after prolonged cultivation in broth and in the absence of bacteriophage, has taken place, the resistant strain W-Little R₁₄, which remains avirulent and resistant to lysis on the first subculture in broth (Protocols V, B and VI, B), tends to become as virulent as the parent culture of M. T. II, from which it originally was derived.^a

In order to ascertain more closely the relationship between the return of virulence and the susceptibility to lysis of the culture, the experiment was repeated and the test of susceptibility, as well as of virulence, was made daily.

^a It should be noted incidentally that simultaneously with these changes in virulence and susceptibility to lysis, the appearance of growth on broth changed from being agglutinated in the first subculture from agar to the diffuse growth characteristic of the parent culture M. T. II.

Pro. ol V.
Virulence of the Reverted W-Little R₁₄ by Mouth.

	A										B										C										
	Original M. T. II										W-Little Ru (resistant to lysis)																				
	18 hr. culture on broth										Grown 18 hrs. on broth										Subjected to 20 passages on broth										
Approximate No. of bacteria given by mouth in .5 cc. volume.....	5,000,000										5,000,000										5,000,000										
No. of mice.....	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	
Time of death after feeding in days.....	14th day																														
	13th "																														
	12th "																														
	11th "																														
	10th "																														
	9th "																														
	8th "																														
	7th "																														
	6th "																														
	5th "																														
	4th "																														
	3rd "																														
	2nd "																														
	1st "																														
No. of survivals.....	3										All 10										5										

For this purpose we returned to the agar culture W-Little R₁₄ (kept on ice all the time), and made a subculture from it into broth. After 18 hours of incubation at 37°C. the resulting broth culture was tested for its virulence and susceptibility to lysis. A little later on the same day, a second subculture in broth was made from the first to be tested next day (after 18 hours of growth) and so on for several days (Protocol VII).

Only the intraperitoneal method of injection was used for testing virulence in this experiment, as it was deemed more convenient, both because of simplicity and the shorter incubation period. When it was observed that the animals died regularly, at a rate approximating that of the mortality of mice injected with the original M. T. II culture, the experiment was interrupted. Results of this experiment indicate that on the sixth transfer in broth the resistant culture W-Little R₁₄ became susceptible to lysis, and at the same time it regained its pathogenicity.

Non-Reversion to Susceptibility of W-178 R₁₄.

As stated in the early part of this paper, resistant strains obtained by us could be roughly divided into two groups, judging by the macroscopic appearance of their respective growth in broth. Since it has been observed further that resistants secured by means of the phage W-Little are susceptible to lysis by the phage W-178 (but not *vice versa*, see Protocol I), we undertook to ascertain whether resistants obtained by the action of the latter "stronger" phage would also undergo reversion to susceptibility and recover virulence if grown in the absence of phage. However, all attempts in this direction have thus far been unsuccessful. The cultures have remained resistant to lysis, and when tested for virulence, after nearly 200 successive passages in broth over a period of 10 months, mice survived an injection of 3,000,000 bacteria intraperitoneally. Throughout the period resistant bacteria exhibited their original characteristics as regards fermentation and antigenic properties, and were found to be free from bacteriophage (not "lysogenic"). Occasionally, when grown on agar plates with a corresponding bacteriophage, some of these cultures gave rise to a few "pale" plaques which were quickly overgrown by resistants. In such cases corresponding broth cultures have, on occasion, shown a slight increase in the titer of the phage, but at

Protocol VII.

Respective Rate of Recovery of Virulence and of Susceptibility to Lysis by the Resistant Subcultures of W-Little R_u.

No. of passages on broth.....	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth
Susceptibility to lysis by W-Little phage.....	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible quickly overgrown	Susceptible but quickly overgrown	Susceptible but overgrown	Susceptible	Susceptible
No. of bacteria given intraperitoneally.....	5,100,000	4,750,000	6,200,000	5,700,000	5,200,000	6,300,000	4,900,000	5,300,000	5,000,000	4,700,000
No. of mice.....	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
14 days										
13 "										
12 "										
11 "										
10 "										
9 "										
8 "										
7 "										
6 "										
5 "										
4 "										
3 "										
2 "										
1 "										

Time of death after injection. in days

* The numbers recorded above were obtained by plating suspensions immediately after injection.

no time was it possible to isolate the few susceptible individuals which must have appeared temporarily in such cultures, to be immediately overgrown by the resistants or to be destroyed by the phage. Since our first observations concerning the behavior of resistants obtained

Protocol VIII.

Attempt to Cause Reversion in the Presence of Antiphage Serum.

No. of transfers previous to test	Culture medium	Character of growth	Susceptibility to lysis as tested	
			In broth	On 1 per cent agar
1	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
2	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
4	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
6	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
7	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
10	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
17	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
25	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—
32	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—

by means of bacteriophage W-178 (18), we have noted a similar failure of resistants to revert to susceptibility when other "strong" phages were used for their production. Although in every instance the resistants were tested and found not to carry phage, we attempted

to grow them in the presence of an antibacteriophage serum, in the hope that we might thus induce a reversion.

In these experiments a resistant strain, isolated by means of a phage P.I.D. and carried for several months without reversion, was employed. Starting from an agar slant culture of this strain P.I.D.-R₁₅, we have made two sets of daily transfers, the first into plain broth and the second into broth containing 0.02 cc. of antibacteriophage serum for each cc. of the medium. From time to time cultures of resistants thus obtained were tested for their susceptibility to lysis in broth as well as in agar. This procedure was continued for a month without leading to reversion, as illustrated on Protocol VIII.

DISCUSSION.

According to the original conception of d'Hérelle and his collaborators, the production of resistants is the result of an increase in the resistance of bacteria against the invasion of bacteriophage. They consider this process analogous to the development of active immunity in higher forms, after exposure to infection (19). In accordance with this theory, resistants might be expected to be more virulent, and actual observation seems to have confirmed the expectations in many instances (2-7). However, our experiments fail to support this notion since, at least in the case of *B. pestis caviæ*, resistants have been found repeatedly to be devoid of virulence. On the other hand, only cultures susceptible to lysis have been found to be pathogenic. In a measure, as cultures of resistants recover their susceptibility to lysis, they again become virulent. The cultures which fail to become susceptible to lysis, under the conditions of the experiment, remain avirulent. The fact that some resistant strains may not become susceptible, after having been carried free from bacteriophage for 200 successive transfers in broth, seems to indicate that the production of resistants is not a phenomenon of active "hereditary" immunization (12), but rather a result of irreversible variation, somewhat analogous to that observed in pneumococcus cultures (13, 14). It has been shown by Arkwright (15), Gratia (16), and others that cultures of bacteria of the colon-typhoid group can be normally dissociated into a number of variants of which at least two are manifestly different in the appearance of their colonies. Gratia (17) found later that the variants normally possess different resistance

to lytic agents and may exhibit varying rates of growth (3). Thus, a bacterial culture may be considered in its cross-section (as indeed any other "population") as composed of individuals approaching a certain type, but occasionally lacking or taking on exaggerated characteristics. Depending upon the conditions of growth, some of the variants may find themselves favored by environment and may become quantitatively dominant. On the contrary, if the environment is changed so that it becomes incompatible with life or the multiplication of a certain type of variant, the latter is eliminated more or less completely, and the whole cross-section of the bacterial population in the culture changes accordingly, with more or less noticeable changes in the biologic activity of the culture as a whole. In the case of a highly specific, "weak" bacteriophage which displays activity only toward a comparatively narrowly defined type of individuals in the culture (as, for example, the phage W-Little), only the most susceptible individuals carrying the potential characteristic of virulence are destroyed, and if such a culture is allowed to grow in the absence of bacteriophage, the few remaining closely related individuals, which may carry the potential characteristic of virulence, begin to multiply anew and to produce the original cross-section of the culture. If, however, a phage of less specificity is used (as, for example, the "strong" phages W-178 or P.I.D.), it may happen that all the individuals carrying the characteristic of virulence are destroyed, and a permanently avirulent culture results.

SUMMARY AND CONCLUSIONS.

Resistants isolated from the overgrowth of cultures of *B. pestis caviæ* (M. T. II) lysed by various strains of specific bacteriophage proved to be avirulent when administered to mice by feeding, or by intraperitoneal injection.

These cultures remained resistant to the action of bacteriophage so long as they were carried on agar. When transferred to broth, however, one group of resistants, namely, those isolated by means of "weak" phages, became susceptible to lysis after five to seven daily passages. The other group of resistants, isolated from the cultures lysed by one of the "strong" phages, failed to become susceptible to lysis even after nearly 200 passages in broth.

Simultaneously with the recovery of susceptibility, the cultures of the first group regained a degree of virulence comparable to that of the parent culture of *B. pestis caviæ*. The cultures of the second group of resistants have failed thus far to recover virulence (10 months after isolation). The latter cultures, apart from lack of both virulence and susceptibility to lysis, are identical with the parent culture of *B. pestis caviæ*, as indicated by biochemical and antigenic properties.

Our findings offer evidence in favor of the view that resistant strains result from selection among variants already existing in the parent culture and do not arise through the inheritance of specific immunity properties produced by the action of phage.

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THE RELATION BETWEEN BODY AND ORGAN WEIGHTS IN THE RABBIT.

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In the previous paper of this series (1), we summarized the results of that part of our study of organ weights the immediate object of which was to define conditions that obtain in normal stock rabbits with respect to mean weights, the tendency to the occurrence of variations in weight, and the probable limits of variation for different organs. As a further contribution to the general problem of physical constitution and as an approach to the study of conditions that determine or affect the weights of organs, we have undertaken an investigation of the normal relation that obtains between body and organ weight and between the weight of one organ and that of another. The purpose of this paper is to report the relation found between body and organ weight as indicated by correlation coefficients and a comparison of group means.

Methods and Material.

The results to be reported are based on data from 645 male rabbits of various breeds. The series contained a few young and a few old animals but the great majority of the animals were between 6 months and 2 years old with about an equal division between those that were sexually mature but not full grown and those that had attained sexual maturity and full growth. All of the animals were in apparent good health but on postmortem examination many of them showed active or healed lesions of some kind. In the present investigation no discrimination was made on the basis of age, breed, or the presence of lesions.

The actual weight and the weight of organs per kilo of net body weight¹ (relative

¹ Net body weight is the gross weight of the animal minus the weight of the gastrointestinal mass as defined in the first paper of this series (Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 69).

TABLE I.

Correlation Coefficients for Actual and Relative Organ Weight with Gross Body Weight.

	Actual	Relative
Net body weight.....	+0.958 \pm 0.002	
Heart.....	+0.755 \pm 0.011	-0.169 \pm 0.026
Testicles.....	+0.555 \pm 0.019	+0.101 \pm 0.028
Kidneys.....	+0.471 \pm 0.021	-0.443 \pm 0.021
Gastrointestinal mass.....	+0.456 \pm 0.021	-0.311 \pm 0.024
Brain.....	+0.451 \pm 0.025	-0.761 \pm 0.013
Suprarenals.....	+0.364 \pm 0.023	-0.066 \pm 0.027
Hypophysis.....	+0.343 \pm 0.024	-0.402 \pm 0.022
Mesenteric lymph nodes.....	+0.307 \pm 0.03	-0.143 \pm 0.032
Liver.....	+0.291 \pm 0.024	-0.301 \pm 0.024
Thyroid.....	+0.248 \pm 0.025	-0.019 \pm 0.027
Deep cervical lymph nodes.....	+0.246 \pm 0.031	-0.058 \pm 0.033
Thymus.....	+0.222 \pm 0.025	-0.160 \pm 0.026
Parathyroids.....	+0.221 \pm 0.025	-0.174 \pm 0.026
Pineal.....	+0.193 \pm 0.026	-0.316 \pm 0.024
Popliteal lymph nodes.....	+0.190 \pm 0.028	-0.271 \pm 0.027
Spleen.....	+0.186 \pm 0.026	-0.113 \pm 0.026
Axillary lymph nodes.....	+0.074 \pm 0.029	-0.321 \pm 0.026

TABLE II.

Correlation Coefficients for Actual and Relative Organ Weight with Net Body Weight.

	Actual	Relative
Gross body weight.....	+0.937 \pm 0.003	
Heart.....	+0.739 \pm 0.012	-0.217 \pm 0.025
Testicles.....	+0.493 \pm 0.021	+0.018 \pm 0.028
Kidneys.....	+0.452 \pm 0.021	-0.473 \pm 0.021
Suprarenals.....	+0.415 \pm 0.022	+0.027 \pm 0.027
Brain.....	+0.392 \pm 0.027	-0.830 \pm 0.010
Hypophysis.....	+0.353 \pm 0.023	-0.449 \pm 0.021
Thyroid.....	+0.261 \pm 0.025	+0.013 \pm 0.027
Mesenteric lymph nodes.....	+0.251 \pm 0.031	-0.222 \pm 0.031
Gastrointestinal mass.....	+0.245 \pm 0.025	-0.448 \pm 0.021
Parathyroids.....	+0.242 \pm 0.025	-0.139 \pm 0.026
Deep cervical lymph nodes.....	+0.228 \pm 0.032	-0.103 \pm 0.033
Liver.....	+0.214 \pm 0.025	-0.417 \pm 0.022
Thymus.....	+0.213 \pm 0.025	-0.171 \pm 0.026
Pineal.....	+0.199 \pm 0.026	-0.323 \pm 0.024
Spleen.....	+0.143 \pm 0.025	-0.166 \pm 0.026
Popliteal lymph nodes.....	+0.116 \pm 0.029	-0.290 \pm 0.027
Axillary lymph nodes.....	+0.063 \pm 0.029	-0.336 \pm 0.026

TABLE III.
Group Means for Body (Gross) and Organ Weights.

Gross body weight	No. of animals	Heart		Testicles		Kidneys		Gastrointestinal mass		Brain	
		Actual gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.
kg.											
1.400	1	1175	3.36	3.22	2.74	20.29	17.28	225.0	191.5	9.38	8.16
1.563	2	1245	3.53	3.74	3.06	11.04	8.87	317.5	261.7	9.38	8.16
1.633	6	1306	3.57	2.27	1.74	10.44	7.97	327.5	252.9	8.18	6.32
1.750	20	1415	4.08	3.09	2.20	11.17	7.90	335.7	245.9	8.44	6.04
1.840	36	1472	4.27	3.80	2.53	11.76	7.99	365.1	250.6	8.60	5.80
1.933	56	1553	4.62	3.74	2.41	11.98	7.73	380.0	244.4	8.87	5.69
2.033	89	1629	4.86	4.15	2.53	12.20	7.52	401.6	248.7	8.86	5.46
2.134	83	1717	5.02	4.16	2.45	12.46	7.26	415.2	243.6	9.23	5.39
2.235	78	1811	5.11	4.72	2.61	12.71	7.04	420.0	233.9	9.28	5.12
2.338	75	1908	5.37	5.02	2.60	13.01	6.84	439.0	225.7	9.26	4.87
2.436	59	2005	5.73	4.99	2.48	13.77	6.88	431.1	217.2	9.27	4.63
2.531	44	2070	5.71	5.58	2.70	13.68	6.62	457.3	222.2	9.43	4.54
2.638	23	2160	5.94	5.29	2.40	13.67	6.32	474.3	220.6	9.24	4.29
2.732	25	2272	6.42	5.87	2.58	14.28	6.27	445.4	197.1	9.76	4.27
2.815	13	2282	6.35	6.19	2.73	14.43	6.33	527.3	233.0	9.82	4.30
2.936	11	2440	6.85	6.52	2.66	16.44	6.74	485.0	200.3	9.65	3.91
3.047	9	2562	7.03	6.43	2.52	15.53	6.11	485.2	187.5	10.06	3.95
3.107	7	2605	7.12	6.33	2.69	15.81	6.65	497.0	191.9	9.39	4.09
3.200	2	2530	7.45	7.30	2.69	16.20	6.41	670.0	265.0	11.30	4.51
3.350	4	2855	7.74	7.74	2.72	16.48	5.82	470.0	166.3	10.28	3.66
3.450	1	2925	7.20	8.58	2.93	15.10	5.16	525.0	179.5	11.03	3.77
3.500	1	3010	7.68	8.98	2.98	15.78	5.23	490.0	162.8		
Correlation coefficient		+0.958 ±0.002	-0.169 ±0.026	+0.555 ±0.019	+0.101 ±0.028	+0.471 ±0.021	-0.443 ±0.021	+0.456 ±0.021	-0.311 ±0.024	+0.451 ±0.025	-0.761 ±0.013

TABLE III—*Concluded.*

Gross body weight	No. of animals	Suprarenals		Hypophysis		Mesenteric lymph nodes		Liver		Thyroid		Deep cervical lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.400	1	0.228	0.194	0.030	0.0255			55.0	46.8	0.175	0.149		
1.563	2	0.505	0.3955	0.0255	0.0208			83.0	68.9	0.1625	0.1311		
1.633	6	0.275	0.2097	0.0237	0.0175	3.03	2.38	61.3	46.8	0.1725	0.1321	0.0988	0.0772
1.750	20	0.3222	0.2259	0.0264	0.0187	2.67	1.92	75.9	54.1	0.2864	0.2024	0.1114	0.0782
1.840	36	0.3285	0.2204	0.0259	0.0176	2.48	1.67	76.5	52.2	0.1866	0.1263	0.1252	0.084
1.933	56	0.3238	0.2083	0.0253	0.0164	3.18	2.05	80.1	51.7	0.1897	0.1216	0.1414	0.0914
2.033	89	0.3239	0.1984	0.0269	0.0166	3.34	2.06	82.3	50.6	0.1944	0.1194	0.1558	0.0959
2.134	83	0.3339	0.1944	0.0273	0.0159	3.26	1.91	82.0	48.0	0.217	0.1266	0.1458	0.0858
2.235	78	0.3694	0.2038	0.0272	0.0150	3.70	2.04	83.4	47.0	0.2079	0.1149	0.1428	0.0785
2.338	75	0.4226	0.2203	0.0282	0.0148	3.38	1.79	92.5	47.4	0.2269	0.1186	0.1484	0.0787
2.436	59	0.4332	0.2164	0.0307	0.0154	3.55	1.79	89.6	44.9	0.2468	0.1221	0.1629	0.0809
2.531	44	0.4358	0.2104	0.0301	0.0142	3.61	1.75	91.0	44.1	0.3095	0.1467	0.1758	0.0851
2.638	23	0.4239	0.1964	0.0311	0.0144	3.95	1.84	84.0	43.7	0.2507	0.1154	0.1678	0.0781
2.732	25	0.4436	0.1947	0.0301	0.0133	4.09	1.79	91.2	40.2	0.2835	0.1239	0.2024	0.0884
2.815	13	0.4117	0.1801	0.0314	0.0138	3.82	1.68	96.9	42.8	0.3202	0.1394	0.2511	0.1108
2.936	11	0.5868	0.2405	0.0319	0.0130	4.19	1.69	92.0	37.8	0.3905	0.1587	0.1969	0.0796
3.047	9	0.5177	0.2006	0.0315	0.0123	4.03	1.57	93.8	36.8	0.2611	0.1019	0.1979	0.0777
3.107	7	0.7000	0.2996	0.0343	0.0142	4.65	1.80	101.6	39.1	0.355	0.1509	0.1238	0.0598
3.200	2	0.475	0.1877	0.0365	0.0144	8.10	3.23	94.5	37.4	0.2075	0.0821	0.185	0.0739
3.350	4	0.436	0.1517	0.0302	0.0106	3.83	1.36	114.8	40.6	0.3775	0.1343	0.2083	0.0753
3.450	1	0.635	0.217	0.032	0.0109	4.00	1.37	120.0	41.0	0.436	0.149	0.225	0.0769
3.500	1	0.590	0.1967	0.050	0.0100	4.85	1.61	90.0	29.9	0.235	0.0784	0.125	0.0416
Correlation coefficient		+0.364 ±0.023	-0.066 ±0.027	+0.343 ±0.024	-0.402 ±0.022	+0.307 ±0.03	-0.143 ±0.032	+0.291 ±0.024	-0.301 ±0.024	+0.248 ±0.025	-0.019 ±0.027	+0.246 ±0.031	-0.058 ±0.033

Gross body weight	No. of animals	Thymus		Parathyroids		Pineal gland		Popliteal lymph nodes		Spleen		Axillary lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.400	1	1.280	1.090	0.010	0.00851	0.015	0.01276	0.180	0.1532	1.15	0.978	0.150	0.1276
1.563	2	2.103	1.720	0.015	0.01213	0.0135	0.011	0.230	0.1715	1.165	0.915	0.130	0.097
1.633	6	1.553	1.197	0.0115	0.00887	0.0163	0.01253	0.184	0.1421	0.787	0.603	0.115	0.0891
1.750	20	1.889	1.334	0.0136	0.00952	0.0144	0.01016	0.208	0.1473	0.753	0.528	0.1497	0.1063
1.840	36	1.991	1.291	0.0161	0.00791	0.0153	0.01041	0.232	0.1546	0.925	0.629	0.1667	0.1138
1.933	56	2.110	1.356	0.0141	0.00735	0.0148	0.0095	0.243	0.1565	0.943	0.602	0.1592	0.1019
2.033	89	2.234	1.375	0.01208	0.00744	0.0152	0.00931	0.252	0.1552	0.954	0.590	0.1687	0.1034
2.134	83	2.269	1.334	0.01197	0.00699	0.0155	0.00906	0.256	0.1492	1.042	0.609	0.1646	0.09597
2.235	78	2.240	1.236	0.0119	0.00661	0.0151	0.00828	0.261	0.1435	0.930	0.519	0.1824	0.1034
2.338	75	2.317	1.224	0.01343	0.00705	0.0158	0.00828	0.261	0.1377	1.044	0.550	0.1739	0.0915
2.436	59	2.356	1.175	0.01338	0.00671	0.0159	0.00793	0.263	0.1312	1.061	0.533	0.1805	0.0896
2.531	44	2.527	1.221	0.01495	0.00726	0.0179	0.00871	0.258	0.1254	1.170	0.572	0.1714	0.0832
2.638	23	2.740	1.272	0.01308	0.00607	0.0161	0.00749	0.285	0.1319	1.013	0.469	0.1794	0.0831
2.732	25	2.657	1.172	0.01356	0.00598	0.0163	0.00716	0.250	0.1100	1.181	0.522	0.164	0.0721
2.815	13	2.689	1.181	0.01346	0.00593	0.0155	0.00683	0.272	0.1200	1.249	0.552	0.1646	0.0734
2.936	11	2.599	1.066	0.01454	0.00593	0.0155	0.00632	0.256	0.1039	1.195	0.489	0.1511	0.0614
3.047	9	2.626	1.026	0.01511	0.00592	0.0197	0.00771	0.304	0.1198	1.246	0.487	0.1661	0.0653
3.107	7	2.739	1.054	0.02143	0.00917	0.021	0.00809	0.279	0.1052	1.502	0.574	0.173	0.0664
3.200	2	2.350	0.928	0.017	0.00671	0.022	0.00871	0.250	0.0999	1.895	0.749	0.175	0.0695
3.350	4	2.530	1.094	0.01625	0.00564	0.0205	0.00716	0.338	0.1195	0.995	0.350	0.2217	0.0776
3.450	1	3.100	1.060	0.012	0.0041	0.018	0.00616	0.320	0.1094	1.300	0.445	0.215	0.0735
3.500	1	1.890	0.630	0.032	0.01064	0.025	0.00834	0.470	0.1561	0.930	0.309	0.180	0.0598
Correlation coefficient		+0.222	-0.160	+0.221	-0.174	+0.193	-0.316	+0.190	-0.271	+0.186	-0.113	+0.074	-0.321
		±0.025	±0.026	±0.025	±0.026	±0.026	±0.024	±0.028	±0.027	±0.026	±0.026	±0.029	±0.026

TABLE IV.
Group Means for Body (Net) and Organ Weights.

Net body weight	No. of animals	Gross body weight	Heart		Testicles		Kidneys		Suprarenals		Brain	
			Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.163	2	1475	3.89	3.34	3.82	3.30	15.26	13.09	0.264	0.228	9.38	8.16
1.247	4	1681	3.93	3.15	2.94	2.37	10.20	7.91	0.288	0.223	8.34	6.69
1.358	14	1733	3.81	2.80	2.62	1.92	11.14	8.19	0.326	0.240	8.53	6.26
1.443	38	1827	4.33	3.0	4.89	3.34	11.42	8.17	0.308	0.211	8.72	6.06
1.550	87	1964	4.66	3.0	3.92	2.52	12.09	7.81	0.313	0.202	8.85	5.70
1.650	90	2067	4.87	2.95	4.21	2.52	12.62	7.66	0.328	0.200	9.07	5.50
1.745	79	2151	4.98	2.85	4.09	2.34	12.34	6.93	0.349	0.200	8.96	5.13
1.844	87	2260	5.24	2.83	4.96	2.69	13.05	7.07	0.372	0.202	9.46	5.11
1.952	70	2382	5.46	2.79	5.14	2.64	13.02	6.69	0.451	0.232	9.18	4.70
2.046	59	2470	5.72	2.80	5.07	2.45	13.51	6.60	0.434	0.212	9.24	4.53
2.146	44	2596	5.86	2.73	5.48	2.54	13.79	6.42	0.427	0.197	9.41	4.38
2.243	23	2729	6.41	2.85	5.78	2.60	14.10	6.47	0.414	0.185	9.58	4.28
2.350	13	2796	6.86	2.93	6.48	2.76	14.66	6.25	0.559	0.238	9.66	4.10
2.461	12	2945	6.97	2.83	6.72	2.73	16.12	6.53	0.448	0.182	9.95	4.04
2.544	9	3044	6.51	2.77	6.34	2.77	15.55	6.56	0.583	0.254	10.01	4.45
2.655	9	3110	7.11	2.68	6.59	2.48	16.13	6.11	0.681	0.257	9.65	3.62
2.870	1	3300	7.34	2.56	9.12	3.15	18.13	6.31	0.475	0.166	9.70	3.38
2.942	3	3392	7.47	2.54	7.56	2.74	14.30	4.85	0.540	0.183	10.62	3.62
3.010	1	3500	7.68	2.55	8.98	2.98	15.78	5.23	0.590	0.197		
Correlation coefficient		+0.937 ±0.003	+0.739 ±0.012	-0.217 ±0.025	+0.018 ±0.028	+0.493 ±0.021	+0.452 ±0.021	-0.473 ±0.021	+0.415 ±0.022	+0.027 ±0.027	+0.392 ±0.027	-0.830 ±0.010

Net body weight	No. of animals	Hypophysis		Thyroid		Mesenteric lymph nodes		Gastrointestinal mass		Parathyroids	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
g.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.163	2	0.029	0.02492	0.1675	0.144			312.0	269.8	0.0125	0.01073
1.247	4	0.0245	0.01858	0.1513	0.1217	3.42	2.73	434.0	348.9	0.0113	0.009
1.358	14	0.0251	0.01849	0.1712	0.1264	2.38	1.74	375.0	276.1	0.0104	0.0077
1.443	38	0.0258	0.01791	0.2343	0.1632	2.97	2.06	381.0	267.2	0.0128	0.0089
1.550	87	0.0254	0.01682	0.185	0.1195	3.20	2.07	412.0	264.8	0.0112	0.0072
1.650	90	0.0274	0.01662	0.2101	0.1273	3.33	2.02	413.0	250.5	0.0129	0.0079
1.745	79	0.0269	0.01544	0.2089	0.1199	3.39	1.94	405.0	232.3	0.0118	0.0068
1.844	87	0.0278	0.01511	0.2096	0.1139	3.53	1.91	414.0	225.1	0.0122	0.0066
1.952	70	0.0286	0.01465	0.2262	0.1151	3.53	1.81	425.0	217.7	0.0137	0.0071
2.046	59	0.0306	0.01496	0.2688	0.131	3.31	1.62	423.0	206.8	0.014	0.0069
2.146	44	0.0303	0.01418	0.3021	0.1406	3.60	1.68	448.0	208.8	0.0139	0.0065
2.243	23	0.0291	0.01299	0.2723	0.1213	4.33	1.93	475.0	211.8	0.012	0.0054
2.350	13	0.031	0.0133	0.2943	0.1679	4.18	1.69	432.0	184.0	0.0125	0.0053
2.461	12	0.0324	0.01318	0.3977	0.1332	4.24	1.72	478.0	192.2	0.0165	0.0067
2.544	9	0.0335	0.0139	0.3733	0.1579	4.42	1.74	504.0	198.2	0.0172	0.0075
2.655	9	0.033	0.01244	0.3272	0.1232	4.14	1.56	448.0	168.7	0.0179	0.0064
2.870	1	0.035	0.0122	0.490	0.1716	2.72	0.95	430.0	149.8	0.016	0.0055
2.942	3	0.030	0.0102	0.3137	0.1067	4.60	1.56	450.0	153.0	0.0166	0.0057
3.010	1	0.030	0.00997	0.235	0.0784	4.85	1.61	490.0	162.8	0.032	0.0106
Correlation coefficient		+0.353 ±0.023	-0.449 ±0.021	+0.261 ±0.025	+0.013 ±0.027	+0.251 ±0.031	-0.222 ±0.031	+0.245 ±0.025	-0.448 ±0.021	+0.242 ±0.025	-0.139 ±0.026

TABLE IV—*Concluded.*

Net body weight kg.	No. of animals	Deep cervical lymph nodes		Liver		Thymus		Pineal gland		Spleen	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.163	2	0.0938	0.0752	83.5	72.1	1.867	1.613	0.0150	0.0129	0.940	0.806
1.247	4	0.1008	0.0745	74.8	59.5	1.345	1.073	0.0155	0.0124	0.753	0.601
1.358	14	0.1285	0.0891	72.2	53.1	1.744	1.285	0.0138	0.0102	0.848	0.640
1.443	38	0.1392	0.090	81.2	56.3	2.014	1.392	0.0144	0.0100	0.886	0.613
1.550	87	0.164	0.0996	82.4	53.1	2.024	1.310	0.0144	0.0093	0.955	0.620
1.650	90	0.1447	0.0833	83.2	50.3	2.268	1.381	0.0158	0.0096	1.021	0.616
1.745	79	0.1437	0.0779	80.8	46.3	2.245	1.291	0.0159	0.0092	1.022	0.588
1.844	87	0.152	0.0777	88.2	47.6	2.279	1.242	0.0153	0.0083	0.963	0.525
1.952	70	0.161	0.0787	87.3	44.7	2.334	1.196	0.0161	0.0082	1.053	0.539
2.046	59	0.1754	0.0817	89.5	43.8	2.587	1.267	0.0166	0.0081	1.100	0.535
2.146	44	0.2196	0.0981	91.2	42.5	2.526	1.175	0.0164	0.0077	1.140	0.530
2.243	23	0.1781	0.0756	96.0	42.6	2.816	1.255	0.0153	0.0069	1.030	0.458
2.350	13	0.2205	0.0895	86.9	36.9	2.514	1.070	0.0166	0.0071	1.078	0.458
2.461	12	0.166	0.0656	90.2	36.6	2.648	1.077	0.0188	0.0077	1.058	0.423
2.544	9	0.1933	0.0726	90.5	35.6	2.285	0.897	0.0189	0.0074	1.334	0.525
2.655	9	0.150	0.0527	96.3	36.3	2.282	1.153	0.0184	0.0069	1.551	0.586
2.870	1	0.1875	0.0639	115.0	40.1	2.050	0.714	0.0200	0.0070	0.900	0.313
2.942	3	0.125	0.0416	106.7	36.3	2.763	0.940	0.0210	0.0071	1.093	0.372
3.010	1			90.0	29.9	1.890	0.630	0.0250	0.0083	0.930	0.309
Correlation coefficient		+0.228 ±0.032	-0.103 ±0.033	+0.214 ±0.025	-0.417 ±0.022	+0.213 ±0.025	-0.171 ±0.026	+0.199 ±0.026	-0.323 ±0.024	+0.143 ±0.025	-0.166 ±0.026

Net body weight kg.	No. of animals	Popliteal lymph nodes		Axillary lymph nodes	
		Actual gm.	Relative gm.	Actual gm.	Relative gm.
1.163	2	0.180	0.1532	0.150	0.1276
1.247	4	0.1725	0.1377	0.1388	0.1109
1.358	14	0.2418	0.1769	0.1527	0.1118
1.443	38	0.2311	0.1569	0.1607	0.1111
1.550	87	0.239	0.1551	0.1568	0.101
1.650	90	0.2546	0.1543	0.1705	0.1033
1.745	79	0.2583	0.1483	0.1659	0.0951
1.844	87	0.2639	0.143	0.1813	0.0984
1.952	70	0.2577	0.1319	0.1756	0.0897
2.046	59	0.2738	0.134	0.1845	0.090
2.146	44	0.2439	0.1135	0.1563	0.0728
2.243	23	0.2264	0.1232	0.1733	0.0773
2.350	13	0.2512	0.1068	0.160	0.0679
2.461	12	0.2845	0.1154	0.1702	0.0694
2.544	9	0.2225	0.0878	0.1292	0.0511
2.655	9	0.2681	0.0997	0.1584	0.0595
2.870	1	0.350	0.122	0.180	0.0627
2.942	3	0.3475	0.1182	0.2775	0.0945
3.010	1	0.470	0.1561	0.180	0.0598
Correlation coefficient		+0.116 ±0.029	-0.290 ±0.027	+0.063 ±0.029	-0.336 ±0.026

weight) were correlated with both gross and net body weight. The correlation coefficients are recorded in Tables I and II in the order of the magnitude of the coefficients obtained for actual weight. Space does not permit the publication of complete correlation tables but a summary of the group means is given in Tables III and IV arranged on the basis of increasing gross and net body weights respectively. The results for gross body weight are plotted in Text-fig. 1; those for net body weight do not differ sufficiently to warrant reproduction. In order to facilitate direct comparison the values for organ weight are plotted on the basis of a percentage deviation from the mean weight of the organ concerned for a given increase in body weight so that the scales of all curves are comparable. The significant parts of the curves are included between heavy perpendicular lines. The groups to the right or left of these lines contained only a few animals but the values are given as they were used in calculating the coefficients.

RESULTS.

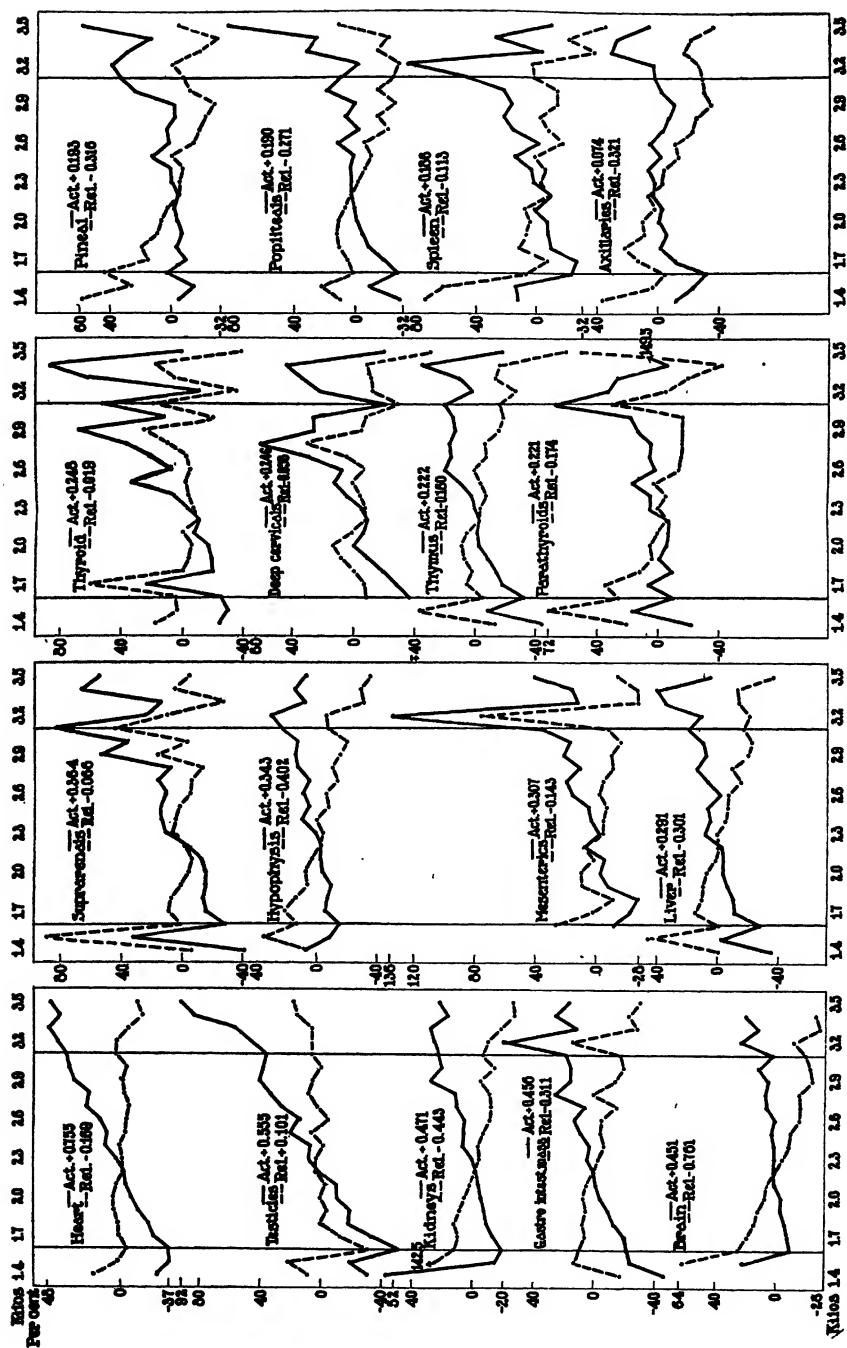
The relations found between body and organ weights are presented in Tables I to IV and Text-fig. 1.

DISCUSSION AND CONCLUSIONS.

The results presented in Tables I to IV and Text-fig. 1 bring out a number of important points concerning the physical constitution of mature and apparently healthy stock rabbits. With very few exceptions, the ratio of the correlation coefficients to their probable errors (Tables I and II) is sufficiently large to warrant acceptance of the results as valid measures of the relation existing between body and organ weight, irrespective of the magnitude of the coefficients.

The coefficients show a great diversity of relations. In the first place, it will be seen that there is a *positive* relation of some kind between the actual weight of all organs and the body weight of the animal while the coefficients for relative weight are either *negative* or *approach a zero order*. That is to say, there is evidence of a common tendency on the part of all organs to weigh more or less according to the weight of the animal but, in only a few instances, is the difference in weight of such an order as to maintain a constant relation between body and organ weight; the weight of the organ per kilo of body weight varies and, as a rule, diminishes as the weight of the animal increases.

In estimating the degree of the correlation there are two values to



TEXT-Fig. 1. Relation of actual and relative organ weights to body weight plotted as a percentage deviation of the group means for organ weight from the mean values for all animals. (Act. = actual organ weight, Rel. = relative organ weight.)

be considered, first, the direct correlation between the actual weight of the organ and that of the body and, second, the inverse relation between the weight of the organ per kilo of body weight and the weight of the body. By reference to Tables I and II, it will be seen that there is no constant relation between these two values. The ideal condition of a high positive and a low negative coefficient, or a coefficient of the zero order, is shown by very few organs. In some cases this relation is reversed while in others the two sets of coefficients are of a comparable magnitude. If, however, we arrange the organs according to the magnitude of the correlation coefficient; for actual weight with gross body weight, as in Table I, it will be seen that in general the so called major organs of the body show the highest values and the lymphoid organs the lowest with the endocrine glands occupying an intermediate position.

The situation presented by correlating relative organ weight with either gross or net body weight is entirely different. The coefficient obtained in this way serves as an inverse measure of the extent to which the increase in the actual weight of different organs approximates the ideal condition of the maintenance of a constant ratio between body and organ weight. In most instances the magnitude of the coefficient for relative weight is smaller than that for actual weight.

Similar conditions obtain when the weights of organs are correlated with net instead of gross body weight (Table II). The chief difference between the two sets of results is in the magnitude of the correlation coefficients for different classes of organs. By correlating actual organ weight with net body weight, the coefficients for the endocrine glands are increased while those for all other organs are either diminished or unaffected; in the case of relative weights, all values are increased with the exception of those for the thyroid, parathyroids, suprarenals, and testicles. While in most instances the change in the magnitude of the coefficient is comparatively small, the effect of this method of correlation is to strengthen the direct correlation of the endocrine glands and to weaken that of other organs.

A clearer conception of the form as well as the measure of the relation between body and organ weight may be gained by an examination of the curves in Text-fig. 1 which give the results obtained by

plotting the group means for actual and for relative organ weight against the corresponding values for gross body weight (Table III). These curves show three distinct forms of relation: first, a linear increase in actual weight which is directly proportional to body weight; second, a straight line increase in actual weight which is of such an order as to produce a linear decrease in the weight of the organ per kilo of body weight; third, an increase in the actual weight of the organ over the lower ranges of body weight with the maintenance of a constant or diminishing level of actual weight over the middle and upper ranges which produces first an increase and then a decrease in the weight of the organ per kilo of body weight (popliteal and axillary lymph nodes).

Other curves appear to be modifications or combinations of these with the possible exception of the curve for the suprarenals. There is some indication that the change in the weight of the suprarenals is not of the order of a uniform progression but shows a diphasic condition characterized by stabilization of actual weights at successive levels or by a succession of increases and decreases in relative weight. It may be that this condition is referable to some factor other than body weight which, in the case of the suprarenals, is sufficiently potent to obscure the influence of the body weight factor.

Analyzing the results obtained from the standpoint of the correlation coefficients and the form of the relation shown, it will be seen that there are comparatively few organs the weights of which are closely related to body weight. The heart shows the closest correlation from every point of view. The coefficient for actual weight is much larger than that of any other organ while the coefficient for relative weight is comparatively small. This, in itself, suggests that there is a constant ratio between the weight of the heart and the weight of the body which holds for practically all ranges of weight within the limits of these observations. This conclusion is borne out by plotting the mean values for actual and relative weights (Table III) against body weight. The curve obtained (Text-fig. 1) shows a linear increase in the actual weight of the heart which is of such an order as to maintain the weight per kilo at a practically constant level. This may be taken as an example of almost perfect physical correlation between the weight of an organ which performs a mechanical function and that of the body which it serves.

The testicles show a similar relation to body weight, giving a high correlation coefficient for actual weight and a small positive coefficient for relative weight, while the mean values (Table III) form a curve which shows the same relation between actual and relative weights on the one hand, and the weight of the body on the other, as the curve for the heart. This result may be regarded as highly significant and indicates that the generally recognized relation between testicular development and growth is of a very high order.

Among the organs studied, the testicles and the heart are the only ones that show a high correlation of this type. The kidneys, the gastrointestinal mass, and the brain come next in the order of magnitude of the correlation coefficients between actual and gross body weight. But, these organs show a negative coefficient for relative weight of a high order which would lead one to infer that the rate of increase in the actual weight of the organ is not proportional to the increase in body weight. By plotting the mean values (Table III, Text-fig. 1), we find that, within the limits of these observations, the increase in the weight of the kidneys is fairly uniform but the rate of increase is such that the weight per kilo of body weight diminishes at a rate which is approximately equal to that of the increase in actual weight, giving correlation coefficients of essentially the same magnitude ($+0.471 \pm 0.021$ and -0.443 ± 0.021). The gastrointestinal mass behaves in much the same manner but the coefficient for relative weight is smaller ($+0.456 \pm 0.021$ and -0.311 ± 0.024), and the regression in the weight of the mass per kilo of body weight is correspondingly less.

The coefficients for the liver ($+0.291 \pm 0.024$; -0.301 ± 0.024) are of a lower order than those for the kidneys and gastrointestinal mass but they show a similar relationship to body weight with a suggestion of a tendency toward an accentuation of the inverse relation which is brought out so strikingly in the case of the brain. The condition presented by the brain is the reverse of that shown by the organs mentioned above. The coefficient for actual weight is $+0.451 \pm 0.025$ while that for relative weight is -0.761 ± 0.013 . These values would lead one to expect a very uniform but very small increase in the actual weight of the brain with increasing body weight. The curve formed by the group means (Table III; Text-fig. 1) shows

a slight and very uniform increase in the actual weight of the brain for animals weighing between 1500 and 2000 gm.; from 2000 to 2500 gm. it is doubtful whether there is any change but in still heavier animals there is apparently a second increase of the same order but less uniform than the first.

The organs considered above form a small group with comparatively close and clearly defined relations to body weight. At the opposite end of the scale, we find such purely lymphoid organs as the popliteal and axillary lymph nodes with negative coefficients that are distinctly larger than the positive coefficients for actual weight. The relation shown by these organs is of a comparatively low order and even this may be an effect of age rather than weight.

It will be seen that the mesenteric and deep cervical lymph nodes differ from the popliteal and axillary nodes; the coefficients for actual weight are larger and the negative coefficients are smaller so that the relation of these two masses of lymphoid tissue to body weight is not only closer than that of the superficial lymph nodes but is also of a different character (Text-fig. 1).

The conditions shown by the spleen and thymus are of especial interest. Their weights appear to be only slightly affected by body weight; the coefficients for actual weight are comparatively small but are slightly larger than those for relative weight so that the results agree with the superficial lymph nodes in one respect and with the deep lymph nodes in another. The closest analogy, however, is with the parathyroids which have coefficients that are almost identical with those of the thymus. This is of interest as we have additional evidence of a relation between these organs.

The endocrine glands show a variety of conditions. The suprarenals and the thyroid give results which differ chiefly with respect to the magnitude of the coefficients. The coefficients for the actual weight of the suprarenals are comparatively large while those for relative weight are very small giving a value for the direct relation which is considerably higher than that of any other organ in this group. The significant feature of the relation shown by these two organs is, however, the constancy of the weight per kilo of body weight within certain limits (Text-fig. 1).

The hypophysis and pineal gland show a relation to body weight

which is the reverse of that shown by the suprarenals and thyroid. In the case of the hypophysis, both sets of coefficients are large but the negative value is larger than the positive so that the reduction in the weight of the organ per kilo of body weight is more clearly defined than the increase in the actual weight of the organ. The pineal gland exhibits a similar tendency with an even greater discrepancy between positive and negative values. The brain and the superficial lymph nodes are the only other organs that show such a decided preponderance of the inverse over the direct relation between body and organ weight.

As has been pointed out, the parathyroids give results that are more nearly comparable to those of the thymus than to other endocrine glands so far as correlation coefficients are concerned. Both sets of coefficients are small and, while the curves for mean weights are decidedly irregular, neither the actual nor the relative weight of the organs appears to be materially affected by body weight. In fact, if we disregard the upper and lower ends of the curve, the results obtained show the nearest approximation to a neutral equilibrium that is given by any of the organs studied.

The significance of the points brought out by this discussion may be made clearer by tabulating the results for gross body weight in the following manner:

Organ	Coefficient		Organ	Coefficient		Organ	Coefficient	
	Actual	Relative		Actual	Relative		Actual	Relative
Heart.....	+0.755	-0.169	(Testicles)	+0.555	+0.101	Deep		
(Testicles).	+0.555	+0.101	Thyroid..	+0.248	-0.019	cervicals..	+0.246	-0.058
			Supra-			Mesen-		
			renals...	+0.364	-0.066	terics....	+0.307	-0.143
Gastro-								
intestinal								
mass....	+0.456	-0.311	Parathy-					
Kidneys...	+0.471	-0.443	roids....	+0.221	-0.174	Spleen....	+0.186	-0.113
Liver.....	+0.291	-0.301				Thymus...	+0.222	-0.160
Brain.....	+0.451	-0.761	Hypo-					
			physis...	+0.343	-0.402	Popliteals..	+0.190	-0.271
			Pineal....	+0.193	-0.316	Axillaries..	+0.074	-0.321

This arrangement of organs takes into account structural and functional relationships as well as the correlation between body and organ weight. The organs studied are divided into three main groups; each of these groups contains three subdivisions, which differ with respect to the relative magnitude of the coefficients for actual and relative weights, arranged in the order of a diminishing direct or increasing inverse relation. The table may be read in any direction. In general, it will be seen that the magnitude of the coefficients for corresponding subdivisions, and hence the closeness of the relation between body and organ weight, diminishes from left to right; in like manner, the direct relation, which is strongest in the first subdivision of each group, diminishes and then changes to an inverse relation. The extreme conditions are represented by the upper left and the lower right divisions.

Tabulation of the results on the basis of the coefficients for net body weight, as has been pointed out above, merely accentuates the direct relation of the endocrine glands and the inverse relation of other organs with only a few minor changes in the actual arrangement of the organs in any given subdivision.

A number of interesting deductions may be drawn from this study. As has already been pointed out, the relations between body and organ weight are diverse. There are a number of organs that show a comparatively high and undoubtedly significant correlation with body weight but only a few that show a correlation of a very high order. In some cases it is the direct relation that is significant, in others, the indirect relation overshadows the direct and, in still other instances, the two are of about equal rank. There are only two clearly defined instances, however, of a direct relation between the actual weight of an organ and that of the body which is sufficiently close to maintain a constant ratio between body and organ weight. In most cases, the relations are such as to favor a diminishing weight per kilo of body weight. For animals weighing less than 2200 to 2300 gm., the weight of the organ exceeds the mean value for animals of all groups while beyond this point the weight becomes less than the mean (Text-fig. 1) so that, as a rule, the larger the animal the smaller the mass of organ tissue per unit of body weight that is available to perform a given function. The amount of the reduction in propor-

tion to body weight varies with different organs, but, as a matter of interest, it may be pointed out that, in several instances the correlation coefficients for actual weight give a rough approximation of the part of the organ that is supposed to be essential to the performance of its function, so far as such information is available, or that the coefficient is the reciprocal of the fractional part of the organ or tissue that may be removed without causing serious impairment of function.

Finally, attention should be called to the fact that the results reported above do not represent conditions that obtain in strictly normal rabbits of a given age and breed. They are reported with a realization that not only these but still other factors may have affected the values obtained. There is, however, substantial evidence that the relations found between body and organ weight have an important bearing on the problem of physical constitution and that the results have both an anatomic and a functional significance. In general, it appears that organs that are related anatomically or that may be supposed to perform analogous or related functions give results of a comparable nature, both with respect to the magnitude and the form of the relation shown.

SUMMARY.

Data from 645 normal rabbits were used as the basis of an investigation of the relation existing between body and organ weights. Actual and relative weights were correlated with both gross and net body weight.

The results obtained varied with different classes of organs but it was found that, in general, there was an agreement between the form and degree of the correlation shown and the structural and functional properties of the organs concerned.

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THE OCCURRENCE AND TREND OF SPONTANEOUS VARIATIONS IN ORGAN WEIGHTS OF NORMAL RABBITS.

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It is generally recognized that the weights of organs, in proportion to body weight, show wide variations in animals of a given species, that they may differ in the two sexes, and that the ratio of organ to body weight changes with age or development. It has also been found that the weights of some organs, notably the endocrine glands, may be affected by other factors such as dietary and climatic conditions and by sexual activity and that some of these conditions tend to produce periodic or even cyclic changes in weight. These conceptions have been derived chiefly from observations on small groups of animals, studied under various conditions, and, usually, with especial reference to a particular organ or to the influence of some particular condition, so that at the present time there is no clearly defined conception of what this series of variations represents in mature animals of a given sex from the standpoint of consecutive changes affecting the animal organism as a whole over long periods of time. This aspect of the subject of organ weight is, however, of the foremost importance in the study of almost any phase of the problem of physical constitution.

In previous papers (1) we have reported results of weight determinations on a large group of normal rabbits with especial reference to mean values, distribution frequencies, constants which, in general, define the variability of organ weights, the probable limits of variation, and the relations that obtain between the weight of the animal and the weight of organs. Thus far, we have dealt primarily with normal standards but we have emphasized the fact that standard values must be used with extreme caution due to the occurrence of

wide variations in weight, the time of occurrence and extent of which are unpredictable and for which the causes are still unknown.

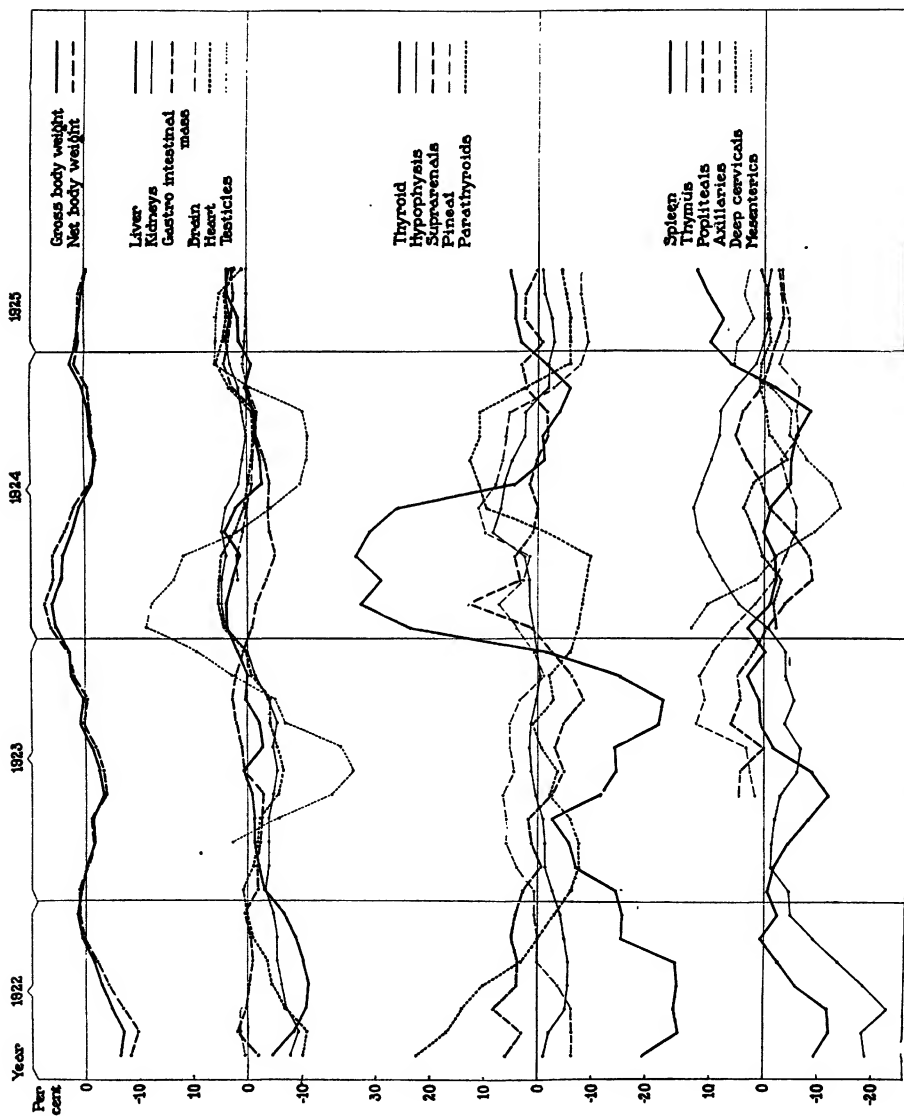
As a means of providing a background for the consideration of more detailed aspects of the problem of the occurrence of variations in organ weight, it seemed desirable to approach the subject with a view to determining first, whether any significant variations in weight of an orderly character could be demonstrated and second, the general course of the variations observed. The purpose of this paper is, therefore, to report the results obtained from a study of the general trend of consecutive variations in mean organ weights of normal male rabbits over a period of $3\frac{1}{2}$ years. The details of the changes observed from month to month and the cause of the variations in weight will not be considered at this time.

Methods and Material.

The material on which this report is based and the methods of investigation that were employed in collecting the data have been described in previous papers (2). We have used results of weight determinations from 645 male rabbits killed in small groups between January 1, 1922, and July 1, 1925. The animals of different groups were fairly comparable as to source, age, and breed; they were given a uniform diet throughout the period covered by the observations but it is not unlikely that the quality of the diet varied and there were some irregularities in the length of time different groups of animals were caged and held under observation before the final weight determinations were made.

The results are presented in the form of a table (Table I) and a series of text-figures (Text-figs. 1 and 2). The actual weights of organs and the weights per kilo of net body weight are recorded in Table I on the basis of the mean values of 39 monthly groups arranged in consecutive order.¹ From these data we have plotted a series of (partially) smoothed curves (Text-figs. 1 and 2) which show the trend of variations in weight from January, 1922, to June, 1925, inclusive, in the form of percentage deviations from the mean normal value. The method of smoothing that was employed is that of a moving weighted average using six monthly groups as the weighting unit according to the following formula:

¹ During the first few months of this investigation, a number of animals that had been used for some minor experimental test was included in the series for normal weight determinations. Subsequently, the data from all of these animals were excluded. In this way all results for July, 1922, were eliminated and the number of animals in other groups was considerably reduced, apparently without materially affecting the results.



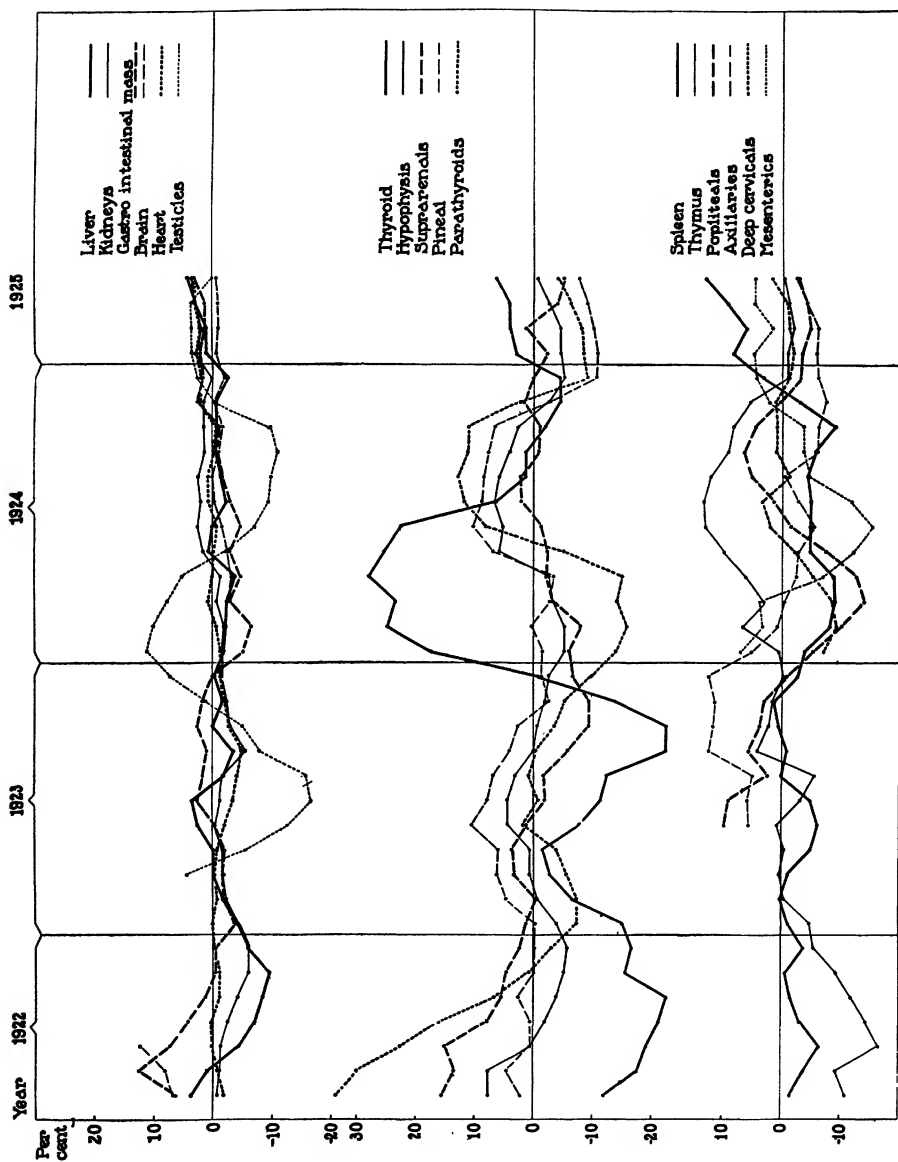
TEXT-FIG. 1. Variations in body and actual organ weight in terms of the percentage deviation from the mean value.

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TEXT-FIG. 2. Variations in the weight of organs per kilo of net body weight in terms of the percentage deviation from the mean.

$\frac{m_1 + m_2 + m_3 + m_4 + m_5 + m_6}{N}$ in which m represents the weighted total for a given month and N the total number of animals for the 6 months period. By this method, major variations are brought out clearly and at the same time some indication of short period variations is retained which we regard as desirable in order to avoid giving an erroneous impression of smooth or perfect continuity.

RESULTS.

The results obtained and the data on which they are based are presented in Text-figs. 1 and 2 and Table I.

DISCUSSION AND CONCLUSIONS.

The results recorded in Table I and Text-figs. 1 and 2 bring out a number of important points concerning the weights of given organs in normal animals, the relation of the weight of one organ to that of others, and the occurrence of variations in weights and relationships. A critical analysis of any of these points would require reference to data which, as yet, have not been presented, but we may refer briefly to a few conditions that are suggested by the smoothed curves in order to clarify certain features of the general tendency that is shown by these curves.

In the first place, there can be no question as to the occurrence of significant variations in the weights of many organs. During the period covered by these observations, it will be noted that all of the organs studied showed apparent variations in weight which assumed the general form of annual cycles. By reference to the tabulated values for mean weight (Table I), it will be seen, however, that the actual variations in weight from month to month were not as uniform as the curves would indicate and that the exact time of occurrence of maximum and minimum weights were in reality somewhat irregular. For example, it is generally stated that the thyroid of animals is largest during the first 4 months of the year, or during the winter, and the curves for thyroid weights would seem to support this view. The figures in Table I show, however, that this is not strictly correct. Exceptionally low values may be obtained at this period of the year and very high values may be obtained for months during which the thyroid is supposed to be small. Perhaps the most constant condition shown by the thyroid, in this case, is the occurrence of low and

TABLE I.
Mean Values for Actual and Relative Weights.

Month	No. of animals	Gross body weight	Net body weight	Gastrointestinal mass		Heart		Liver		Kidneys		Spleen	
				Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
				gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
<i>1922</i>													
January.....	2	2263	1760	503	284	5.08	2.92	106.0	60.6	12.3	6.94	0.640	0.356
February.....	5	2000	1560	423	272	4.50	2.89	76.6	48.9	10.5	6.75	0.968	0.620
March.....	6	2408	1918	467	246	5.38	2.81	94.5	50.0	12.8	6.74	1.005	0.535
April.....	6	2050	1622	413	260	4.66	2.91	81.9	53.1	12.1	7.52	0.810	0.510
May.....	5	2075	1683	381	227	4.71	2.83	84.2	50.3	12.9	7.65	0.828	0.490
June.....	12	2050	1654	378	230	4.56	2.75	72.9	44.5	11.6	6.98	1.017	0.621
September.....	10	2090	1579	502	323	4.63	2.97	71.3	45.9	11.0	6.96	0.752	0.459
October.....	17	2229	1814	403	223	5.36	2.96	72.0	39.9	12.5	6.91	0.927	0.515
November.....	14	2464	2025	432	218	5.72	2.85	82.4	41.6	13.0	6.54	1.230	0.620
December.....	15	2290	1882	403	216	5.09	2.70	83.1	44.4	12.3	6.57	1.070	0.576
<i>1923</i>													
January.....	17	2415	1985	417	212	5.69	2.87	84.5	42.8	12.8	6.45	1.105	0.558
February.....	13	2187	1776	407	232	5.01	2.84	85.8	49.1	12.2	6.99	0.820	0.498
March.....	15	2088	1675	413	245	5.04	3.02	92.3	56.4	12.3	7.46	0.940	0.577
April.....	17	2097	1683	406	243	4.81	2.84	76.4	46.3	12.4	7.78	0.876	0.559
May.....	12	2296	1871	414	220	5.44	2.90	88.1	46.9	12.6	6.78	1.050	0.570
June.....	9	2347	1950	397	204	4.89	2.51	82.1	42.3	12.2	6.24	0.773	0.388
July.....	10	2193	1781	412	230	4.71	2.64	86.7	48.1	12.1	6.77	0.941	0.531
September.....	15	2303	1824	479	269	4.77	2.63	92.7	51.3	12.0	6.69	0.988	0.536
October.....	14	2234	1809	423	235	5.34	2.92	76.9	43.1	13.0	7.23	1.340	0.735
November.....	15	2300	1870	427	231	5.12	2.75	77.9	42.5	11.7	6.56	0.976	0.531
December.....	15	2238	1797	431	243	5.38	3.01	98.0	55.1	13.8	7.77	1.058	0.610

1924													
January.....	18	2475	2054	421	214	5.85	2.89	82.7	41.2	13.5	6.76	1.010	0.500
February.....	17	2307	1920	387	208	5.23	2.79	95.1	50.7	13.5	7.17	0.801	0.423
March.....	20	2638	2192	432	201	5.92	2.72	99.6	46.8	14.6	6.75	1.162	0.504
April.....	15	2345	1947	398	212	5.71	2.96	78.4	41.6	13.4	6.96	1.043	0.560
May.....	22	2160	1773	387	222	5.27	3.00	76.8	43.4	12.7	7.25	0.935	0.512
June.....	14	2177	1791	386	220	5.15	2.92	90.4	51.2	13.0	7.40	1.068	0.619
July.....	19	2253	1827	426	236	5.04	2.80	96.2	53.4	14.1	7.93	1.130	0.622
August.....	20	2135	1743	392	234	4.82	2.77	84.3	48.6	13.1	7.58	0.773	0.446
September.....	32	2305	1887	415	223	5.54	2.91	79.8	42.9	12.7	6.80	0.962	0.514
October.....	15	2227	1803	423	238	5.42	3.00	79.4	44.7	12.9	7.22	1.030	0.579
November.....	19	2282	1856	426	232	5.14	2.78	81.2	45.2	12.4	6.71	0.864	0.465
December.....	14	2210	1820	390	217	5.20	2.87	91.9	50.8	13.5	7.44	0.844	0.458
1925													
January.....	45	2332	1851	476	262	5.90	3.08	94.0	51.7	14.0	7.57	1.239	0.669
February.....	40	2422	1983	439	227	5.68	2.88	81.2	42.3	13.7	7.04	1.223	0.636
March.....	27	2169	1771	398	229	5.17	3.01	90.6	52.5	13.4	7.87	1.170	0.673
April.....	30	2246	1833	413	227	5.25	2.88	83.8	46.1	12.5	6.90	0.943	0.523
May.....	20	2199	1792	407	227	5.21	2.91	96.6	53.2	13.2	7.37	1.119	0.636
June.....	14	1959	1545	404	263	4.14	2.67	91.4	58.5	12.2	7.90	1.113	0.720

TABLE I—Continued.

Month	No. of animals	Thymus		Brain		Testicles		Thyroid		Parathyroids		Hypophysis	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
1922													
January.....	2	1.802	1.047	9.63	5.0	6.63	3.75	0.2350	0.1318	0.0200	0.0117	0.0260	0.0149
February.....	5	1.687	1.093	8.54	5.49	5.77	3.69	0.1356	0.0871	0.0134	0.0086	0.0302	0.0195
March.....	6	1.983	1.055	9.32	4.89	6.15	3.18	0.2125	0.1115	0.0173	0.0091	0.0292	0.0154
April.....	6	1.906	1.245	9.47	5.96	6.60	4.05	0.1737	0.1088	0.0168	0.0106	0.0280	0.0176
May.....	5	2.181	1.288	9.31	5.59	4.50	2.64	0.1820	0.1094	0.0134	0.0079	0.0256	0.0152
June.....	12	1.745	1.059	9.49	5.74	4.04	2.47	0.1973	0.1208	0.0159	0.0097	0.0275	0.0167
September.....	10	1.915	1.199	8.60	6.10			0.1306	0.0836	0.0137	0.0088	0.0261	0.0165
October.....	17	1.480	0.824					0.1672	0.0929	0.0132	0.0073	0.0254	0.0141
November.....	14	2.328	1.190					0.1840	0.0936	0.0137	0.0068	0.0271	0.0135
December.....	15	2.414	1.299			6.29	3.52	0.1779	0.0953	0.0111	0.0059	0.0271	0.0146
1923													
January.....	17	2.570	1.324			5.49	2.68	0.2927	0.1465	0.0112	0.0056	0.0274	0.0140
February.....	13	2.423	1.376			4.69	2.62	0.1936	0.1103	0.0132	0.0078	0.0286	0.0163
March.....	15	2.060	1.252			4.61	2.73	0.1681	0.1030	0.0105	0.0063	0.0286	0.0173
April.....	17	1.887	1.132			3.15	1.93	0.2602	0.1504	0.0121	0.0073	0.0276	0.0169
May.....	12	2.313	1.256			4.66	2.47	0.1992	0.1071	0.0143	0.0078	0.0273	0.0147
June.....	9	2.383	1.216			3.65	1.75	0.2174	0.1147	0.0127	0.0065	0.0272	0.0139
July.....	10	2.638	1.481			2.68	1.53	0.1816	0.1026	0.0141	0.0078	0.0301	0.0169
September.....	15	2.006	1.099			3.68	2.03	0.1599	0.0874	0.0122	0.0068	0.0300	0.0165
October.....	14	1.980	1.094			5.23	2.88	0.1683	0.0951	0.0123	0.0069	0.0291	0.0163
November.....	15	2.172	1.736			5.46	2.92	0.1733	0.0924	0.0133	0.0070	0.0269	0.0145
December.....	15	2.088	1.150	9.11	5.37	5.06	2.80	0.1856	0.1047	0.0114	0.0064	0.0261	0.0147

TABLE I—Concluded.

Month	No. of animals	Suprarenals		Pineal		Popliteal lymph nodes		Axillary lymph nodes		Deep cervical lymph nodes		Mesenteric lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
1922													
January.....	2	0.3885	0.2238	0.0120	0.0062							3.65	2.03
February.....	5	0.3228	0.2084	0.0163	0.0107								
March.....	6	0.5193	0.2769	0.0175	0.0092								
April.....	6	0.4165	0.2566	0.0127	0.0080								
May.....	5	0.3556	0.2060	0.0136	0.0081								
June.....	12	0.4032	0.2449	0.0150	0.0093								
September.....	10	0.3545	0.2183	0.0142	0.0091								
October.....	17	0.4366	0.2382	0.0153	0.0085								
November.....	14	0.3868	0.1874	0.0179	0.0089								
December.....	15	0.4020	0.2134	0.0173	0.0094								
1923													
January.....	17	0.4069	0.2057	0.0148	0.0075	0.2700	0.1360	0.1650	0.0830				
February.....	13	0.3816	0.2136	0.0158	0.0091	0.2715	0.1531	0.1790	0.1022				
March.....	15	0.3394	0.2027	0.0148	0.0089	0.3410	0.2065	0.2041	0.1229				
April.....	17	0.3667	0.2194	0.0182	0.0109	0.2440	0.1483	0.1487	0.0897				
May.....	12	0.4392	0.2415	0.0185	0.0099	0.2533	0.1382	0.1929	0.1043				
June.....	9	0.4283	0.2209	0.0177	0.0093	0.2166	0.1109	0.1611	0.0835				
July.....	10	0.2922	0.1589	0.0163	0.0093	0.2552	0.1459	0.1437	0.0818				
September.....	15	0.3333	0.1795	0.0144	0.0080	0.2683	0.1472	0.1863	0.1022				
October.....	14	0.3740	0.2056	0.0152	0.0085	0.2850	0.1599	0.2124	0.1206	0.2092	0.1198	3.48	1.95
November.....	15	0.3357	0.1784	0.0183	0.0099	0.3177	0.1714	0.2230	0.1229	0.1510	0.0789	3.81	1.95
December.....	15	0.3507	0.1943	0.0165	0.0091	0.2355	0.1272	0.1787	0.1013	0.1105	0.0666	4.03	2.23

1924													
18	January.....	0.4277	0.2051	0.0139	0.0069	0.2492	0.1228	0.1819	0.0924	0.1353	0.0671	4.48	2.24
17	February.....	0.3996	0.2008	0.0175	0.0095	0.2173	0.1158	0.1394	0.0965	0.1206	0.0623	4.08	2.14
20	March.....	0.4128	0.1904	0.0174	0.0081	0.2368	0.1099	0.1573	0.0749	0.1894	0.0834	3.62	1.68
15	April.....	0.3653	0.1873	0.0182	0.0095	0.2255	0.1191	0.1619	0.0853	0.2100	0.1050	2.80	1.46
22	May.....	0.4000	0.2309	0.0145	0.0083	0.2325	0.1316	0.1782	0.1084	0.1414	0.0812	2.35	1.36
14	June.....	0.3759	0.2115	0.0151	0.0087	0.2484	0.1424	0.1529	0.0873	0.1493	0.0863	3.16	1.79
19	July.....	0.3545	0.2018	0.0214	0.0119	0.2927	0.1628	0.1632	0.0909	0.1452	0.0826	2.97	1.65
20	August.....	0.3779	0.2134	0.0186	0.0111	0.2835	0.1635	0.1422	0.0813	0.1443	0.0828	3.00	1.72
32	September.....	0.4236	0.2233	0.0154	0.0082	0.2547	0.1374	0.1682	0.0892	0.1680	0.0894	3.61	1.94
15	October.....	0.3263	0.1792	0.0166	0.0093	0.2823	0.1583	0.1787	0.0991	0.1323	0.0729	4.09	2.30
19	November.....	0.3521	0.1934	0.0139	0.0076	0.2591	0.1401	0.1484	0.0798	0.1732	0.0925	2.92	1.56
14	December.....	0.3789	0.2074	0.0144	0.0080	0.2214	0.1216	0.1568	0.0862	0.1546	0.0853	3.12	1.72
1925													
45	January.....	0.4143	0.2231	0.0144	0.0078	0.2486	0.1350	0.1580	0.0855	0.1585	0.0856	3.82	2.10
40	February.....	0.3983	0.2003	0.0138	0.0070	0.2550	0.1313	0.1763	0.0889	0.1492	0.0766	3.88	1.99
27	March.....	0.3399	0.1945	0.0144	0.0082	0.2352	0.1365	0.1594	0.0925	0.1546	0.0875	3.46	2.00
30	April.....	0.4283	0.2348	0.0159	0.0088	0.2498	0.1365	0.1693	0.0937	0.1497	0.0815	3.27	1.79
20	May.....	0.3500	0.1962	0.0150	0.0085	0.2665	0.1475	0.1663	0.0930	0.1600	0.0950	3.54	1.98
14	June.....	0.2736	0.1794	0.0142	0.0092	0.2366	0.1526	0.1605	0.1017	0.1782	0.1130	2.76	1.80

comparatively uniform values during the months of September, October, and November, and even in December, rather than the occurrence of high values from January or December to April inclusive. Moreover, it will be seen that the thyroid showed a late spring or early summer increase in weight with as much regularity as it did the more generally recognized winter increase which, after all, may be comparatively slight and of irregular occurrence and duration, with intervening periods during which the weight of the organ is small.

These conditions are mentioned in order to make it clear that, while there is an evident tendency to the occurrence of cyclic variations in the weights of many organs with distinct seasonal relations, these changes do not occur with perfect regularity or in perfect conformity with the (astronomical) progression of the seasons. At times, the departure from the usual course is very decided.

A second point to be noted is the variation in the magnitude of the change from year to year. This is again illustrated best by the thyroid which shows that, in addition to the annual cycle, there was a progressive increase in the magnitude of the values obtained over a period of at least 3 years. Scattered observations made during the latter part of 1921, which are not included in the results recorded above, indicate that the maximum weight of the thyroid for the winter of 1921-22 was approximately the same as that given for the winter of 1922-23; it may have been a little higher or a little lower. During the winter of 1924-25, the weight was lower than for the preceding winter but it was still high (see Table I) and, while systematic observations were discontinued in June, 1925, sufficient data have been accumulated to make it certain that the weight of the thyroid increased during 1925-26 to a much higher level than any hitherto recorded by us. This applies to rabbits raised in our own laboratories as well as to those obtained from the usual outside sources which shows a widespread prevalence of a condition that might even be termed a goitrous enlargement.

If all of these observations are taken into account, the results obtained suggest the occurrence of a second cycle of weight variation extending over a period of years. The indications are that our observations may have been started at or near a period of minimum

thyroid weight (a period of maximum weight for some organs) or at the turning point of a decreasing phase of a cycle and that most of our observations fell within the limits of an increasing phase, the maximum of which we are now approaching or have reached during the past few months.

A third feature of the results to which attention may be directed is the suggested relation of the change in the weight of one organ to that of other organs. This is also shown best by the relation of the curves for the thyroid to those for other members of the endocrine system but it will be seen that still other organs show something of the same relation to the thyroid. In general, it may be said that the weight curve for the thyroid stands alone while those for the other endocrines tend to group themselves together and to move in a direction opposite to that of the thyroid with a definite lag in the movements. Still, these relations are not entirely constant. At times, all of the curves are more or less parallel and move in the same direction or they display irregularities in their movements. There is, however, a definite suggestion of a coordination of movements and apparently the manner in which this coordination is effected is influenced to some extent by the actual as well as the relative positions of the organs with respect to the mean normal or zero line. Thus, during 1922 and the early part of 1923 there was an apparent tendency for the weights of all organs to converge toward the mean normal. For a time thereafter, weights were comparatively stable while movements were more or less parallel and in the same direction. But, with the subsequent marked increase in the weight of the thyroid, the curves for other organs continued their downward course, initiating a series of reverse movements which were very decided and clearly defined during the greater part of 1924. This series of changes was followed in time by a second period of comparative stability and parallel coordination of movements which differed in some respects from that of 1923. This apparent variation in the coordination of weights at different levels with respect to the mean normal illustrates the difficulty of attempting to deduce a general rule governing the relation of one organ to another.

Finally, attention may be called to the great difference in the magnitude of the variation in weight shown by different organs.

The changes exhibited by the heart, brain, kidneys, liver, and gastrointestinal mass are of an entirely different order from those of the endocrine glands, the testicles, and lymphoid tissues. This raises the question of the significance of the variations shown and, while it is not our intention to enter into a general discussion of this aspect of the subject at the present time, it seems desirable to refer briefly to the possible influence on organ weight of certain factors that are inherent in the material, namely, the age and weight of animals.

The curves for gross and net body weight in Text-fig. 1 show periodic variations similar to those of the curves for organ weight. The variations in these curves represent not only differences in body weight but differences in age as well and are due to unavoidable conditions of animal supply. For example, during the winter months the supply of fully mature animals is more abundant than at any other time; as the supply of such animals becomes depleted, it is necessary to use a larger proportion of younger and smaller animals during the spring, summer, and early fall. These conditions are repeated year after year; they are reflected in the body weight curves and undoubtedly do have an effect on the curves for organ weight.

The factors of age and weight are so intimately bound up with each other that we cannot attempt to make any distinctions at this time but some indication of the influence that might be expected from this combination of factors may be obtained from the correlation coefficients for organ and body weight (3) which were calculated without distinction as to age so that they are directly applicable to the present instance. On this basis, one would expect that the body weight factor would affect the weights of the heart, testicles, kidneys, gastrointestinal mass, and brain more than other organs as their correlation coefficients are larger, and a careful comparison of the curves will show that the curves for actual and relative weights of these organs do conform most closely with those for body weight. Moreover, the degree of conformity is roughly proportional to the magnitude of the correlation coefficients. The gastrointestinal mass forms an exception and the extent of the variation shown by the testicles is out of proportion to that of other organs in this group.

It is evident, therefore, that, in the case of the organs mentioned, the body weight factor is of considerable importance and may indeed

account for the greater part of the variation shown. The liver appears to be less affected by this factor and a comparison of the curves for the endocrine glands and lymphoid tissues with those for body weight shows that the magnitude of the values and the movements of the curves are virtually independent of those for body weight. Still, it must not be assumed that the body weight factor had no influence on the results obtained for these organs as some of them show a fairly close correlation with body weight. On the whole, the results are in agreement with what might be expected on the basis of the correlation coefficients and it seems that, in the case of these organs, the influence of body weight was of subsidiary importance. There is some evidence, however, that, at times, the influence of this factor may have been such as to alter or mask the effects of other conditions and this possibility should not be overlooked.

It would appear, therefore, that the results recorded above give a composite picture of variations in weight due largely to external causes. At the present time, we are not concerned with point to point variations or with the exact magnitude of the change in weight shown but with the general course of events and the occurrence of changes that are so clearly defined and of such proportions that their significance cannot be questioned. The course of events represented by this series of observations may not agree in all respects with what might have been found had it been possible to follow the weights of organs in a given group of animals over the same period of time, but the manner in which the investigation was carried out and the nature of the results obtained are sufficient to warrant the conclusion that the picture presented gives a fair conception of changes in weight and weight relationships that actually occurred at this particular time.

If we consider these results from the point of view of normal standards, it is at once apparent that no fixed standard of weight or relation can be established. Conditions that prevail at one time differ greatly from those found at another and thus far we have no acceptable basis for making discriminations as to the normality of the findings for one period as compared with another. It seems that the animal organism is subject to the influence of extraneous conditions which determine the relations that obtain and the changes in

the weights of organs that must occur from time to time in order to maintain the health of the animal under changing conditions of life. Hence, weights and relations that would be regarded as normal for one period might be decidedly abnormal for another. Recognition of the occurrence of variations in weight as an essential attribute of organs and a knowledge of the extent to which the weights of various organs may be affected by environmental conditions is of fundamental importance in the study of problems of physical constitution from either an anatomic or functional point of view and is deserving of especial emphasis as the tendency at the present time is to stress standardization and conformity to rule rather than the occurrence of variations.

SUMMARY.

Records of organ weights of normal rabbits covering a period of $3\frac{1}{2}$ years were analyzed with a view to determining first, whether any significant variations of an orderly character could be demonstrated and, second, the general trend of the variations that occurred.

It was found that many organs showed decided variations in weight which assumed the form of definite annual cycles. In addition, there was some evidence of a second cycle covering a period of years. These changes were most marked in the case of the endocrine glands (including the testicles), the lymphoid organs, and the liver.

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ETIOLOGY OF OROYA FEVER.

III. THE BEHAVIOR OF *BARTONELLA BACILLIFORMIS* IN *MACACUS RHESUS*.

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PLATES 22 TO 24.

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As reported in a previous paper,¹ a strain of *Bartonella bacilliformis* isolated from the blood of a fatal case of Oroya fever when inoculated into young *Macacus rhesus* monkeys induced a protracted course of intermittent fever, resembling that observed in some human cases of Oroya fever. The microorganism was demonstrated in the red cells of these animals and recovered in culture, but the clinical manifestations in monkeys differed from those of the human disease in that none of the animals showed the severe anemia so characteristic of Oroya fever. It was also shown that intradermal inoculation of the cultures into the shaved skin of the eyebrows of the monkeys gave rise to a granulomatous nodule rich in capillaries, in which the microorganisms could be demonstrated by section and by culture. The nodules presented a marked resemblance to those of verruga peruviana.

Further study of the behavior of *Bartonella bacilliformis* in *rhesus* monkeys, particularly with reference to the effects of passage strains, has brought to light a number of additional facts, which throw some light on the variety of manifestations of *Bartonella* infection in man.

The protocols are presented in chronological order, and the extraordinary variability in response to inoculation of the parasite is illustrated by all of them. The severity of infection in most instances is referable probably to enhanced virulence of the parasite through adaptation to the animal. In Monkey 5 we have an example of severe systemic infection induced by local inoculation. In Monkey 6 intra-

¹ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

venous and intraperitoneal inoculation gave rise to a fatal systemic infection, which was not accompanied by local lesions, and which, except for the slight degree of anemia present, resembled a case of simple Oroya fever; the fever was severe (104–105°C.) and continuous, and the parasites were present in the blood in high titer and also in the lymph glands, spleen, and bone marrow. Monkey 7 was inoculated intradermally and intravenously with both passage virus and cultures, and the reaction was constitutionally as well as locally severe; *Bartonella bacilliformis* was isolated from blood diluted 1:10,000,000, from nodular tissue diluted 1:10,000, and also from the lymph glands, spleen, and bone marrow. Monkey 8, on the other hand, while receiving considerable amounts of passage virus both locally and intravenously, manifested no local and only mild systemic symptoms. Monkey 18 is of special interest as an illustration of a severe type of verruga such as may result from the inoculation of monkeys with human verruga tissues,^{2,3} while the striking fact brought out in the case of Monkey 25 is the simultaneous occurrence of severe symptoms of both verruga and Oroya fever, typical verruga nodules appearing spontaneously at sites remote from those of local inoculation. These appearances had not been observed by previous investigators in monkeys inoculated with human verruga tissues.

Quantitative estimates of the number of *Bartonella bacilliformis* present at any given time in the blood are expressed in terms of the highest dilution of the blood from which cultures of the organism could be obtained. The culture method is the only satisfactory test of the presence of the parasite. The use of monkeys is obviously not practicable for quantitative determination and would not, moreover, be reliable because of the variation in susceptibility to *Bartonella* infection. Careful microscopical examination of stained blood films was not, of course, neglected, but when one considers that citrated blood of as high titer as 1:100,000 would show only 1 cell invaded by the parasite among 3,000 cells (1 cc. of citrated blood containing approximately 3 billion red cells), the difficulties of this method of demonstrating the organism become evident.

² Jadassohn, G., and Seiffert, G., *Z. Hyg. u. Infektionskrankh.*, 1910, lxi, 247.

³ Mayer, M., Rocha Lima, H., and Werner, H., *Munch. med. Woch.*, 1913, lx, 739.

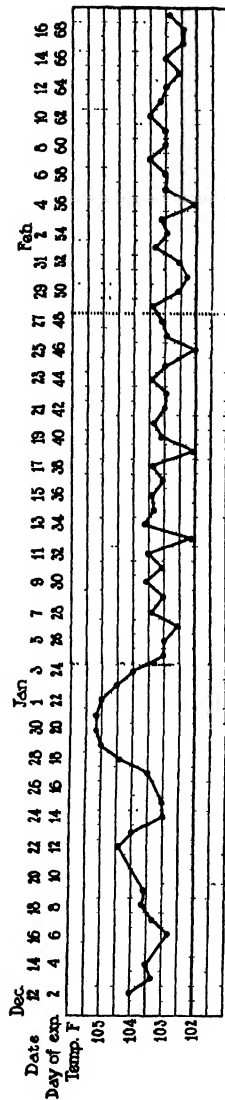
The Invasion of the Blood by B. bacilliformis after Intradermal Inoculation.

The ease with which *Bartonella bacilliformis* may enter the general circulation after intradermal inoculation is illustrated in the case of *M. rhesus* 5.

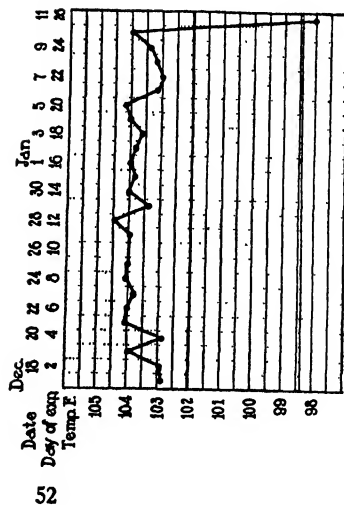
M. rhesus 5 (Text-fig. 1). A young monkey, about 2,000 gm.—a size which has been found most satisfactory for experimental work with *Bartonella bacilliformis*—was inoculated, Dec. 11, 1925, intradermally on the shaved left eyebrow, with 0.2 cc. of a saline suspension of pooled cultures which on dark-field examination showed 3 to 4 motile microorganisms per field. The mixture included 1 cc. of condensation water from a 9 day old culture on a blood agar slant, derived from the original human blood, and 0.3 cc., in 20 cc. of 0.9 per cent saline, of a 10 day old culture, derived from the blood of *M. rhesus* 1, and grown on leptospira medium at 25°C. Cultures made with the blood withdrawn on that date (11 days after inoculation) revealed the presence of *Bartonella bacilliformis* in a titer of 1:100,000, and in stained preparations the parasites were demonstrated in small numbers in the erythrocytes. Cultures made on Dec. 28, 1925, and on Jan. 5, 12, and 25, 1926, also yielded positive results. The animal showed the highest fever during this period, the temperature being continuously at 105°F. from Dec. 29 to Jan. 1. The last two specimens of blood had a titer of only 1:10, and the parasites could no longer be demonstrated in stained smears, hence the number of organisms in the blood stream had diminished rapidly within a period of 24 days. The site of intradermal inoculation on the left eyebrow, however, began to show induration nearly 42 days after injection; on Jan. 22 it was about 2 × 3 mm. in area and was slightly raised; on Feb. 1 it measured 6 × 6 mm., and on Feb. 4 about 9 × 9 mm. It was at first pale and semitransparent, but firm to the touch; then it became a deep rose-red and finally cherry-like in form and color. During the following 10 to 12 days it remained nearly stationary, but later it showed slight excoriation at the apex and was removed* for transfer, culture, and histological studies. It proved to be infective for *rhesus* monkeys, yielded a pure culture of *Bartonella bacilliformis*, and on section showed the characteristic endothelial proliferation with intracellular localization of the organism.

Blood cultures made on Feb. 9 and 17 yielded growth of *Bartonella bacilliformis* in 0.1 cc. of a 1:10 dilution. At no time after subsidence of the early violent febrile reaction had the animal's temperature been higher than 103.6°F. The animal was killed under ether anesthesia on Feb. 17, 1926, 68 days after inoculation, in order that the distribution of the microorganisms and the character of the specific lesions might be ascertained.

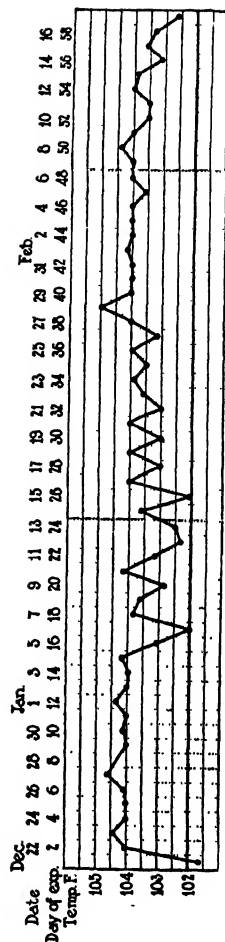
* All operations were performed under ether anesthesia.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

Autopsy.—Spleen enlarged and granular on surface, culture +. Lymph glands everywhere enlarged, culture +. Heart blood culture +. Bone marrow (femur) reddish, culture +. Lungs normal, culture negative. Liver normal, culture negative. Kidney normal, culture lost because of contamination.

With the cooperation of Dr. J. H. Bauer, erythrocyte counts and hemoglobin estimates⁴ were made on several occasions. The results follow:

Jan. 12, 1926 (32 days after inoculation). Erythrocytes 6,169,000. Hemoglobin 82 per cent.

Jan. 25, 1926 (45 days after inoculation). Erythrocytes 5,901,000. Hemoglobin 75 per cent.

Feb. 15, 1926 (66 days after inoculation). Erythrocytes 5,248,000. Hemoglobin 70 per cent.

Feb. 17, 1926 (68 days after inoculation). Erythrocytes 4,856,000. Hemoglobin 70 per cent.

The chronic nature of the infection is clearly indicated by the persistence of the parasites in the peripheral blood, bone marrow, lymph nodes, and spleen. The number of microorganisms demonstrated by stained preparations in the blood corpuscles was never large at any time, even during the febrile or "septicemic" period. There was little sign of anemia until the latter part of the experiment, when the number of red cells seemed definitely to have diminished, notwithstanding the marked fall in temperature and the scarcity of *Bartonella bacilliformis* in the blood at this time.

Variability in the Effect of Inoculation of Bartonella bacilliformis into Macacus rhesus.

The outcome of inoculation of a given pathogenic microorganism must depend in part on the virulence of the strain and in part on the degree of susceptibility of the host. In the case of a newly isolated microorganism the effect of successive animal passages on virulence is problematic; the pathogenic property may be either weakened or enhanced by continued passage. Experiments have just been described in which a second passage of *B. bacilliformis* through *Macacus rhesus* indicated a decided increase in virulence of the organism for this species of animal. The present experiment has to do with the effects of further direct passages in *rhesus* monkeys.

⁴Dr. J. H. Bauer was kind enough to make the blood counts on all the animals of this report. The hemoglobin estimates were made by means of the Sahli hemoglobinometer.

As the protocol shows, intravenous and intraperitoneal inoculation of a young monkey (*M. rhesus* 6) with the citrated blood from infected monkeys of the same species led to an immediate and severe febrile reaction to which the animal succumbed after 3 weeks of continuous fever of about 104°F.

M. rhesus 6 (Text-fig. 2) received 2 cc. of citrated blood from *M. rhesus* 2 intravenously on Dec. 17, 1925, and 5 cc. of a mixture of citrated blood from Monkeys 2, 3, and 4 intraperitoneally on Dec. 18. The temperature rose to 104°F. within 48 hours, dropped to 103° for 1 day, returned to 104°, and remained at about that point until the 20th day. It was 103° on the 21st to 24th days and 104° on the 25th, but on the 26th day it fell suddenly to 98° (subnormal). The animal was cyanotic, cold, and rigid and died during ether anesthesia.

Blood cultures made on the 4th, 12th, 18th, and 26th days after inoculation yielded the following results:

Dec. 21, 1925. Positive with blood diluted 1:100,000.

" 28, " " (untitrated).

Jan. 3, 1926. Positive with blood diluted 1:100,000.

" 11, " " " " " 1:10,000.

" " " " " plasma " 1:10.

The erythrocyte count on Jan. 3, 1926 (17th day), was 4,760,000, hemoglobin 75 per cent. Examination of organs at autopsy revealed enlargement of the spleen and of the lymph glands generally. The other organs appeared normal. *Bartonella bacilliformis* was obtained in pure culture from blood, spleen, and lymph nodes.

The striking phenomena in the foregoing experiment were the absence of localized lesions and the sudden collapse of the animal. The latter can hardly have been due to the insignificant anemia that had developed. The persistent presence of *Bartonella bacilliformis* in large numbers in the blood and organs may have resulted in a toxemia.

That different individual animals react differently to practically the same infective material is demonstrated in the following experiment.

M. rhesus 7 (Text-fig. 3) was inoculated intravenously on Dec. 21, 1925, with 5 cc. of mixed citrate blood from Monkeys 2, 3, and 4. At the same time intradermal inoculations were made on the right eyebrow with 0.2 cc. of an emulsion of the nodule from Monkey 4 and on the left with 0.2 cc. of a young culture, grown on leptospira medium, of blood from Monkey 2. Adjacent areas on each eyebrow were scarified and smeared with the same material which had been injected intradermally into that eyebrow.

In the case of this animal observation was especially directed to (1) the quantitative fluctuation of *Bartonella bacilliformis* in the blood, (2) the course of the local nodular reactions, and (3) the effect of the infection upon the red corpuscles and hemoglobin, this portion of the study being carried out by Dr. Bauer.

The results of blood culture were as follows:

Dec. 24, 1925 (3 days after inoculation). Positive with blood diluted 1:10,000,000.

Dec. 28, 1925 (7 days after inoculation). Positive (not titrated).

Jan. 4, 1926 (14 days after inoculation). Positive with blood diluted 1:1,000,000.

Jan. 18, 1926 (28 days after inoculation). Positive with blood diluted 1:10.

" 29, " (39 " " "). " " " " 1:10.

Feb. 9, " (50 " " "). " (not titrated).

" 17, " (58 " " "). " (" ").

The relatively large number of microorganisms found in the blood withdrawn about 72 hours after intravenous inoculation indicates a strikingly rapid multiplication of the parasites. During the 11 days there would appear to have been a gradual diminution in the number of organisms, and the findings point to an astonishing elimination in the following 2 weeks, since on Jan. 18 *Bartonella bacilliformis* could not be cultivated from blood in dilutions higher than 1:10. The titer was still 1:10, 40 days after inoculation, and the organisms were present in the circulation after 58 days.

In this instance, as in many others, *Bartonella bacilliformis* was demonstrable in small numbers of erythrocytes when the blood titer was high. But as the titer fell to 1:10, examination of film preparations yielded negative results.

The intradermal inoculation of the culture gave rise to a definite nodule (2 × 3 mm.) within 11 days. The lesion gradually increased in size (to 4 × 6 mm.) and had assumed a rose color when it was excised for study on Jan. 8, 1925 (19th day after inoculation). No lesion developed at the site where the culture was inoculated by scarification. The nodule at the site of intradermal inoculation of the nodule emulsion from Monkey 4 became noticeable only after 20 days, and at the same time a small area of induration appeared on the adjacent scarified area. The nodule and indurated area became gradually redder and larger and were removed for study 29 days after inoculation.

fig. 4). During the first 12 days the temperature rose as high as 106°F., and during the second and third periods it fluctuated about 104° for about 10 days. Although the temperature was 104° on the day following inoculation, the febrile reaction seems to have become definite on the 6th day.

Four blood cultures were made during life and one post mortem, with the following results:

Jan. 4, 1926 (5 days after inoculation). Positive with blood diluted 1:10.

" 8, " (9 " " "). " " undiluted blood.

" 18, " (19 " " "). " " blood diluted 1:1,000.

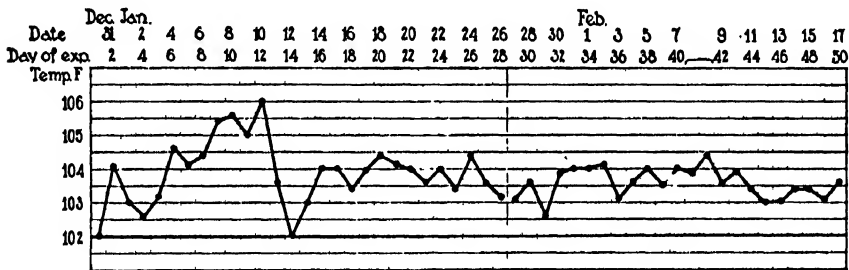
Feb. 3, " (35 " " "). " " " " 1:10.

" 17, " (49 " " "). Negative.

Blood counts showed no anemia:

Jan. 8, 1926 (9 days after inoculation). Erythrocytes 5,506,000. Hemo-
globin 80 per cent.

Feb. 3, 1926 (35 days after inoculation). Erythrocytes 5,414,000. Hemo-
globin 80 per cent.



TEXT-FIG. 4.

Feb. 17, 1926 (49 days after inoculation). Erythrocytes 5,496,000. Hemo-
globin 80 per cent.

The animal was killed by etherization on Feb. 17, 1926, for examination. Autopsy revealed marked enlargement of the lymph glands. No other changes were noticeable. The spleen, blood, and bone marrow failed to yield cultures, but growth was obtained from the emulsion of the lymph nodes.

As a result of the inoculations on the eyebrows, four tiny indurated areas appeared within 9 to 10 days but did not enlarge and within 18 days had practically disappeared. A red spot of pin-point size was noticed on the scarified area after 29 days, but it also disappeared in another week.

While the nodule employed for inoculation in this instance probably contained fewer organisms than the one removed from Monkey 4 earlier in its evolution, yet the suspension from it yielded a pure growth of *Bartonella bacilliformis* in a 1:10 dilution, and a large number

of organisms were found in sections. Loss of virulence may have resulted from the final etherization of Monkey 4.

The comparatively mild infection, accompanied by severe febrile reactions, seems to have been due to a higher resistance of this particular animal, but the chronic nature of the *Bartonella* infection is indicated even in this instance by the persistence of the microorganisms in the lymph glands.

The Reproduction of Striking Clinical Features of Oroya Fever and Verruga.

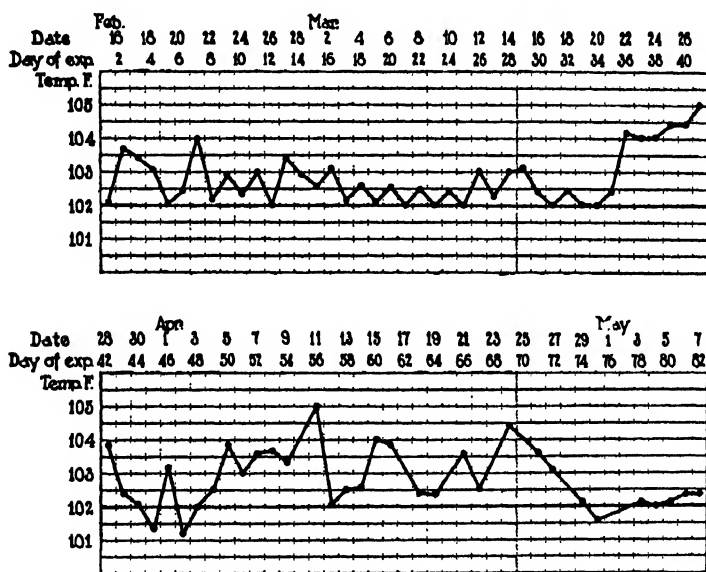
Up to this point in the work, the manifestations by monkeys of infection with *Bartonella bacilliformis* resembled human Oroya fever or verruga in essentials, *i.e.*, in the typical localization of the organism and in the characteristic skin lesions, respectively, but the severe anemia of Oroya fever and the general eruption of verruga had not as yet been reproduced in monkeys.

In Monkey 18, however, the local lesions, while not general or spontaneous, attained extraordinary size and resembled those described by previous investigators as resulting from the inoculation of suspensions of human verruga tissues into monkeys. Monkey 25 developed the spontaneous generalized eruption characteristic of verruga and also severe symptoms of Oroya fever (anemia, presence of *Bartonella bacilliformis* in considerable numbers in the erythrocytes). Although the period of fever was very brief in this animal, the manifestations of infection were similar to those of certain human cases in which Oroya fever and verruga are simultaneously present.⁵

M. rhesus 18 (Text-fig. 5) was inoculated on Feb. 15, 1926, with passage virus (suspension of nodular tissue) from Monkey 5. The shaved left eyebrow received two intradermal injections of the emulsion, and the shaved right eyebrow was scarified and smeared with the same material. The skin of the abdomen was shaved, and on the left side two intradermal injections were made of the emulsion, while on the right two scarified areas (about 3×3 cm. each) were spread with the same material. Induration at the sites of inoculation became evident in about 14 days and were definite after 26 days (Fig. 1). The lesions steadily increased in size, and 36 days after inoculation the largest of the nodules on the scarified areas of the right eyebrow measured 2.5×2 cm. and was 1 cm. high

⁵ Odriozola, E., *La maladie de Carrion*, Paris, 1896.

(Fig. 7), while the two round subcutaneous nodules on the left abdominal wall stood out about 1 cm. and showed a reddish spot at the point of insertion of the syringe needle (Fig. 8). The lesions were of maximum size 59 days after inoculation (Fig. 2) and remained stationary for about 14 days. Retrogression was slow, but at the time of writing (99 days after inoculation) they are pale, fibrous, and considerably decreased in size, as shown in Figs. 3 and 11. No ulceration or softening of the lesions could be detected at any time. The examination of a nodule excised from the right eyebrow 26 days after inoculation revealed the characteristic granulomatous structure with proliferation of endothelial cells (Fig. 9), in the cytoplasm of which were found varying numbers of *Bartonella bacilliformis*, usually in clumps (Fig. 10).



TEXT-FIG. 5.

Bartonella bacilliformis was demonstrated in sections of the lesions and isolated in culture from emulsions of the tissue. It was recovered from the blood of the monkey on the following occasions:

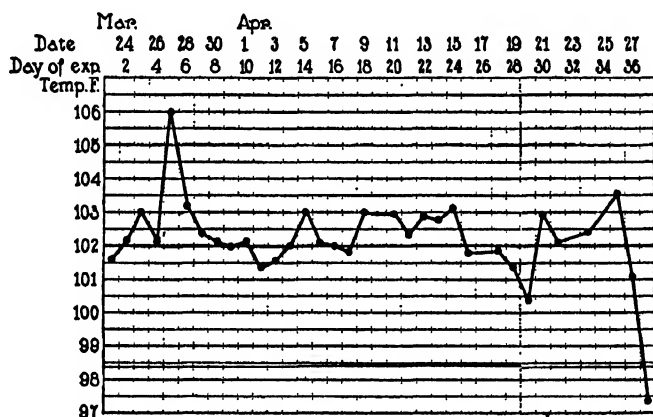
Mar. 5, 1926 (18 days after inoculation). Positive in blood diluted 1:100,000.
 " 18, " (31 " " "). " " " " 1:100,000.
 " 27, " (40 " " "). " " " " 1:10.

Comparison of blood counts made on the day of inoculation and 60 days later show that there was a definite decrease in the number of red cells during the course of illness:

Feb. 15, 1926. Erythrocytes 5,356,000. Hemoglobin 90 per cent.
 Apr. 16, " " 4,120,000. " 60 " "

One of the most striking features of the experimental *Bartonella* lesions is the slow continuous formation of granulomatous tissue on a scarified surface of the skin, which results in an enormous accumulation of endothelial cells and subsequent capillary formation, a process apparently identical with that which takes place in the lesions of verruga in man.

M. rhesus 25 (Text-fig. 6) was inoculated intravenously on Mar. 23, 1926, with 6 cc. of a saline suspension of cultures grown on blood slants for 10 days at 25°C. At the same time the shaved right eyebrow was inoculated intradermally as well as by scarification with a suspension of nodular tissue from *M. rhesus* 18. The left eyebrow was similarly inoculated with culture. This animal had



TEXT-FIG. 6.

only 1 day of high temperature, Mar. 27, when 106°F. was recorded; otherwise the course of disease was practically afebrile.

The first sign of infection in this instance was a peculiar condition of the upper eyelids, the edges of which were irregularly thickened, and perhaps a little pinkish on Apr. 7, 15 days after inoculation. Within a week numerous military, cherry-red nodules appeared around the eyes on both sides, and distinct nodules became noticeable at the site of intradermal inoculation as well as on the scarified areas. The animal presented a striking aspect (Fig. 4). These lesions, and several military nodules scattered over the body, especially near the right inguinal surface (Fig. 5) gradually became larger within the next 5 or 6 days and partly confluent. On the posterior surface of the left leg nodules of varying size appeared within 3 weeks after inoculation (Fig. 6) and remained until death. The eyes were virtually closed 36 days after inoculation (Apr. 28, 1926), when the animal died (Fig. 12). One of the spontaneous nodules was removed for cultural

and histological examination on Apr. 15. It showed the usual endothelial proliferation (Fig. 13), and large numbers of *Bartonella bacilliformis* were present (Fig. 14).

The number of red corpuscles infected with *Bartonella bacilliformis* was larger than in any of the monkeys previously studied, cells invaded by the parasites being readily demonstrable in film preparations. Curiously enough, however, the detection of the organism was not accomplished, even by the culture method, during the 48 hours preceding death.

Mar. 27 (5 days after inoculation). Positive with blood diluted 1:1,000.

Apr. 8 (18 " " "). " " " " 1:100,000.

" 16 (26 " " "). " " " " 1:1,000,000.

The results of blood counts by Dr. Bauer were as follows:

Mar. 27, 1926 (4 days after inoculation). Erythrocytes 5,288,000. Hemoglobin 85 per cent.

Apr. 20, 1926 (28 days after inoculation). Erythrocytes 3,240,000. Hemoglobin 45 per cent.

Apr. 22, 1926 (30 days after inoculation). Erythrocytes 2,992,000. Hemoglobin 40 per cent.

Apr. 24, 1926 (32 days after inoculation). Erythrocytes 3,368,000. Hemoglobin 40 per cent.

Apr. 26, 1926 (34 days after inoculation). Erythrocytes 2,736,000. Hemoglobin 35 per cent.

Apr. 27, 1926 (35 days after inoculation). Erythrocytes 2,120,000. Hemoglobin 30 per cent.

Apr. 28, 1926 (36 days after inoculation). Erythrocytes 1,624,000. Hemoglobin 25 per cent.

Autopsy.—Very much emaciated, wax-yellow. The nodules on the eyebrows remained unopened, but the lesions on the lids had partly ulcerated, and the tissues had become somewhat necrotic. The eyes were almost closed. There was some seropurulent discharge from the ulcerated nodules, which were nearly confluent. Corneæ not affected. Spontaneous miliary nodules were found on the abdomen, thighs, and legs, the posterior surfaces of both legs, in particular, being covered with cutaneous or subcutaneous nodules of varying size (Fig. 6), some adherent to the fascia. Some nodules were hemorrhagic.

There was exudative pericarditis, due to the presence of minute Gram-negative, motile bacilli in enormous numbers, especially within the polymorphonuclear leucocytes. The heart muscles were pale and flabby, but the pleuræ did not appear to be involved. Lungs normal. Liver perhaps enlarged, and pale. Spleen somewhat enlarged and soft. Stomach empty, normal. Intestines and mesentery: General lymphatic system hypertrophied everywhere. Other organs normal. Bone marrow (femur) dark red.

The findings described suggest a terminal secondary bacterial pericarditis, which may have been the result of transfer by the animal

of infective material from the ulcerated eyelid to a needle wound caused by heart puncture. Notwithstanding this late stage invasion, the essential features of the course of illness in this animal were, I believe, the result of local and constitutional infection with *Bartonella bacilliformis*.

SUMMARY.

The experiments reported here were carried on in the main with passage strains of *Bartonella bacilliformis*, and the results indicate that the virulence of the organism has been considerably enhanced by passage through susceptible animals. While the animals of the earlier experimental series showed no anemia, some of the present group manifested a definite reduction in the number of red cells and in hemoglobin, and in one instance (*M. rhesus* 25) anemia was of the extreme type so often associated with Oroya fever in man. The anemic condition appeared to be secondary in character, however, nucleated red cells being few in number. In this animal also *Bartonella bacilliformis* was readily demonstrated in the erythrocytes by means of stained smears, though the number of cells invaded by the parasites was by no means so great as in the human infection.

In most instances of experimental *Bartonella* infection so far induced the demonstration of the parasites by ordinary routine examination of stained film preparations is possible only when the titer of the blood exceeds 1:1,000. Prolonged search of many slides has not been attempted, however. The number of microorganisms in the blood, as shown by culture tests of ascending dilutions, was in most instances highest (1:100,000 to 1:10,000,000) during the early period of the infection coincident usually with the period of highest fever, falling to a titer of 1:10 during the last half of the disease. In one of the fatally infected monkeys, however, the titer increased from 1:10 on the 4th day to 1:1,000,000 on the 24th day. The titer of the blood was equally great in Monkeys 5 and 6, although the former was inoculated locally, the other intravenously and intraperitoneally. The largest proportion of infected red cells was found in Monkey 25, while the blood titer, as shown by culture test, was highest in Monkey 7.

The febrile reaction varied in the animals of this series from a severe continuous fever of 104–105°F., lasting 2 to 3 months, in one instance,

with a remittance during the 3rd to 5th weeks, to the acute high fever (106°F.) of 1 day's duration in the fatally infected monkey, No. 25. The more usual reaction, however, is an irregular course of moderate fever with one or more periods of high temperature (105°).

Bartonella bacilliformis was constantly demonstrated, both microscopically and by culture tests, in the lymph glands of animals sacrificed 2 to 3 months after inoculation, and in two of three instances it was present also in the spleen, bone marrow, and heart blood. In the case of *M. rhesus* 6, which died 26 days after inoculation, the microorganism was obtained also in culture from the lymph glands, spleen, and heart blood taken at autopsy. In the other animal which died, a terminal bacterial infection, while not obscuring the effects of the *Bartonella* infection, made it impossible to isolate the parasite from either blood or tissues.

The skin lesions, whether of the nodular type, induced by introduction of the virus intradermally or by application to the scarified skin, or of the miliary character occurring spontaneously as a result of systemic infection, always yielded cultures of *Bartonella bacilliformis*, and stained sections of such lesions revealed the parasites in large numbers in their characteristic situation in the endothelial cells.

A chronic, systemic infection, in which the lymph glands are enlarged and *Bartonella bacilliformis* is present in the blood in high titer, may be induced by local inoculation, as shown in the case of *M. rhesus* 5.

The local lesions induced in one instance by introduction of a passage strain, both intradermally and by scarification, attained within 2 months extraordinary size, the nodules arising at adjacent sites of inoculation on the right eyebrow having coalesced into a large pedunculated mass which overhung the eye. This type of reaction had not been observed hitherto in the course of the present study but has been described by earlier investigators as a result of the inoculation of monkeys with human verruga tissues.

The striking fact brought out in the present study is the variety of responses to inoculation which animals of the same species may manifest. The clinical features of the infection may be typical of Oroya fever or may resemble those of verruga peruviana, and in *M. rhesus* 25 we have an instance of a type of infection in which the

characteristic phenomena of both conditions are simultaneously present. Whether the appearance will resemble those of the one or the other condition appears to depend on the susceptibility of the individual as well as on the virulence of the organism. Moreover, it seems probable that different degrees of resistance to the invasion of the parasite on the part of the blood cells, internal organs, or skin of a given animal may determine the predominant clinical manifestations of the infection. The factor of variation in susceptibility of different individuals or different tissues of the same individual would account for the variety of types of human *Bartonella* infection.⁶

EXPLANATION OF PLATES.

PLATE 22.

All figures natural size.

FIG. 1. *M. rhesus* 18, 26 days after inoculation. The nodule on the inner aspect of the left eyebrow was subsequently removed for examination.

FIG. 2. *M. rhesus* 18, 59 days after inoculation, when the nodules had reached their maximum development. The nodules on the right side had been induced by inoculation by scarification; there was no softening or ulceration of these lesions at any time. The nodule on the left side was induced by intradermic inoculation.

FIG. 3. *M. rhesus* 18, 99 days after inoculation, when the nodules had retrogressed considerably and the animal was decidedly paler.

FIG. 4. *M. rhesus* 25, 23 days after inoculation. The indurations which had developed at the sites of inoculation on the inner aspect of each eyebrow were almost eclipsed by the numerous spontaneous deep red nodules which had appeared about the eyes.

FIG. 5. *M. rhesus* 25, showing the spontaneous nodules which developed in the inguinal region, 29 days after inoculation.

FIG. 6. *M. rhesus* 25, showing the spontaneous nodules which had appeared on the posterior surface of the legs 29 days after inoculation.

PLATE 23.

FIG. 7. *M. rhesus* 18, 36 days after inoculation, showing the appearance of the lesions at the sites of intradermal inoculation and of inoculation by scarification. Natural size.

FIG. 8. *M. rhesus* 18, 36 days after inoculation, showing the lesions on the abdomen. The verrucous lesions on the right side were induced by scarification,

⁶ Arce, J., La enfermedad de Carrion, Lima, 1920.

the two round subcutaneous nodules on the left side by intradermal and partly subcutaneous inoculation. Natural size.

FIG. 9. Section of nodule removed from eyebrow of *M. rhesus* 18, 26 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 10. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 11. *M. rhesus* 18, 99 days after inoculation, showing the appearance of the receding lesions on the abdomen. One of the nodules at the sites of intradermal inoculation had been removed.

PLATE 24.

FIG. 12. *M. rhesus* 25, at the time of death 36 days after inoculation.

FIG. 13. Section of spontaneous nodule of *M. rhesus* 25, removed 23 days after inoculation. Giemsa stain. $\times 182$.

FIG. 14. The same section at a magnification of 1,000 times, showing the presence of masses of *Bartonella bacilliformis*.



FIG. 1. *M. rhesus* 18,
26 days after inoculation.



FIG. 4. *M. rhesus* 25,
23 days after inoculation.



FIG. 2. *M. rhesus* 18,
59 days after inoculation.

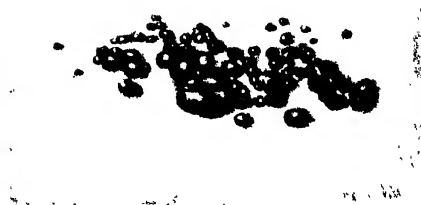


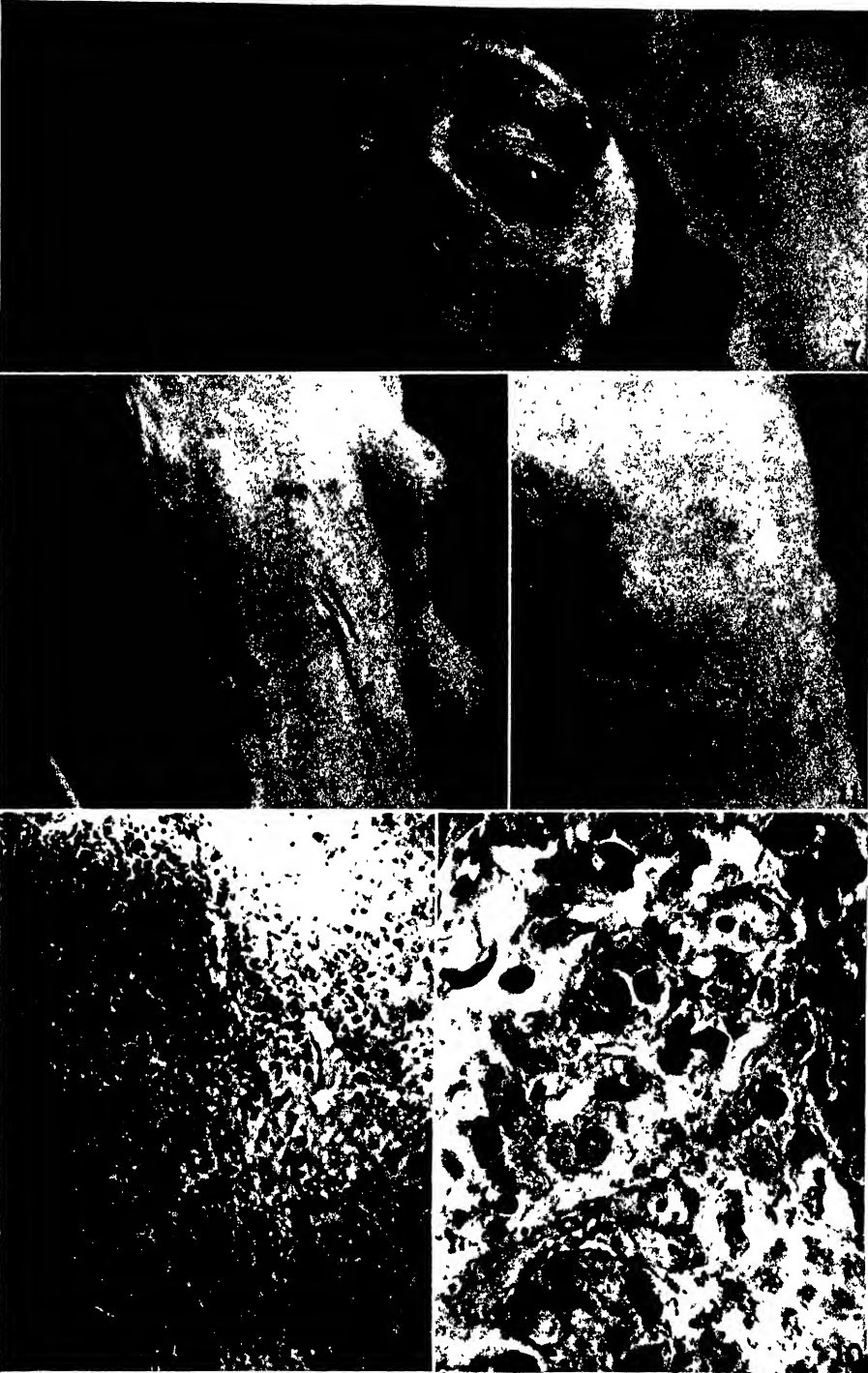
FIG. 5. *M. rhesus* 25
(groin), 29 days after inoculation.



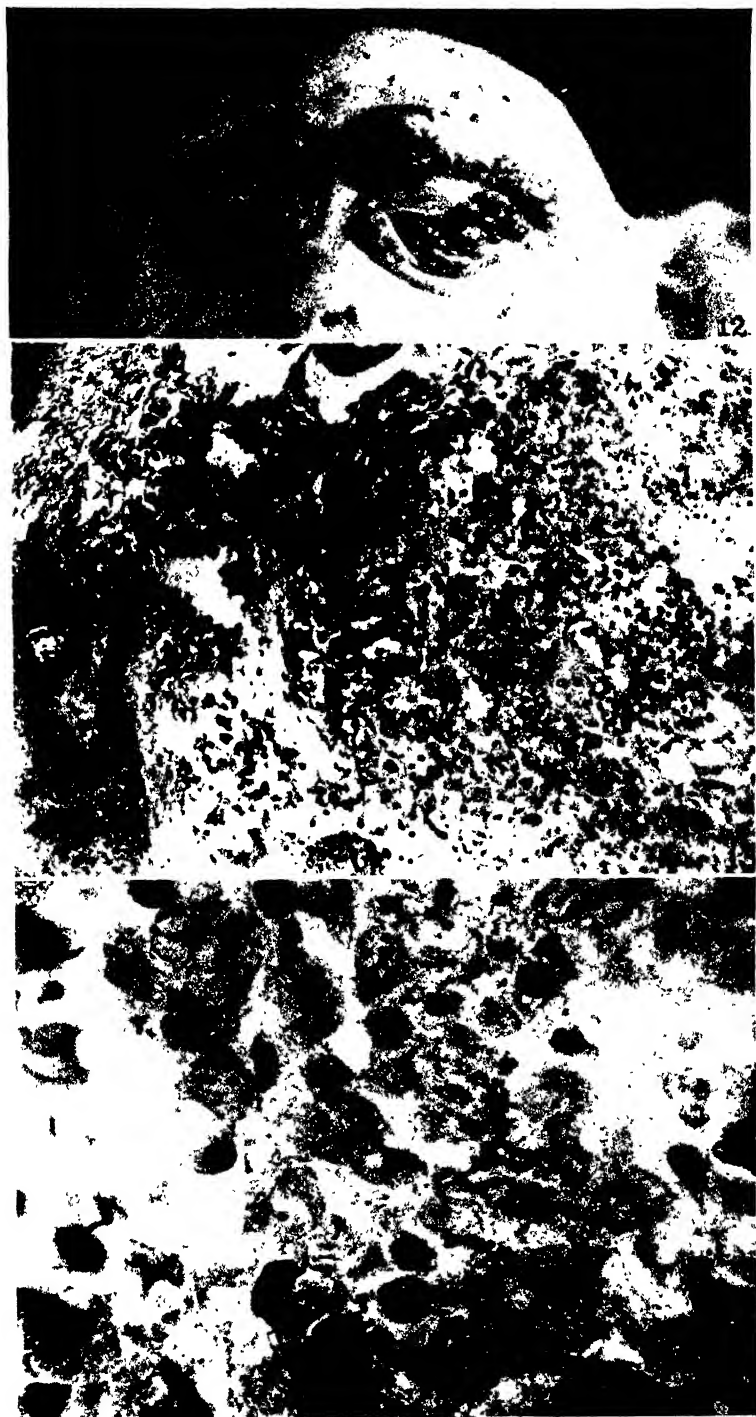
FIG. 3. *M. rhesus* 18,
99 days after inoculation.



FIG. 6. *M. rhesus* 25
(leg), 29 days after inoculation.



(Noguchi: Etiology of Oroya fever. III.)



(Noguchi: Etiology of Oroya fever. III.)

ETIOLOGY OF OROYA FEVER.

IV. THE EFFECT OF INOCULATION OF ANTHROPOID APES WITH BARTONELLA BACILLIFORMIS.

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PLATES 25 TO 28.

(Received for publication, June 18, 1926.)

It has been reported in previous papers that the intravenous inoculation of young *rhesus* monkeys with a strain of *Bartonella bacilliformis* isolated from a case of Oroya fever gives rise to two essential manifestations of Oroya fever, namely, protracted fever, and typical localization of the parasite within the red blood cells (Fig. 1), while the introduction of the organism intradermally induces skin lesions which are indistinguishable from those of *verruca peruviana*.¹ It has been shown also that after intradermal inoculation the organism may pass into the circulation and invade the red blood cells, that it may give rise to an anemic condition comparable with that of Oroya fever, except for the absence of appreciable numbers of nucleated red cells in the blood stream, and that it may induce a generalized eruption similar to that of human *verruca*.² In most instances, however, the inoculated monkeys have shown little or no anemia, and comparatively few erythrocytes are invaded by the parasite, while the *verruca* nodules have usually arisen only at the sites of inoculation into the skin.

Since the invasion of the red corpuscles by the bacilliform organisms is the most characteristic sign of Oroya fever, and the formation of nodules by the proliferation of endothelial cells is equally typical of *verruca*, the findings described indicated that the organism cultivated was probably the cause of both these conditions. Nevertheless, it

¹ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1925, xliii, 851.

² Noguchi, H., *J. Exp. Med.*, 1926, xlv, 697.

was desirable to reproduce, if possible, the complete clinical picture of Oroya fever,—the extreme anemia of pernicious type, and the invasion of large numbers of the erythrocytes by the microorganism, such as takes place in human Oroya fever (Fig. 2). The opportunity presented itself of studying the effect of the inoculation of two anthropoid apes with cultures and passage strains of *Bartonella bacilliformis*, and the experiments were undertaken in the hope that these animals, because of their close phylogenetic relationship to man,^{3,4} might prove sufficiently susceptible to the infection to manifest all of the severe symptoms to which the parasite gives rise in man, either of the type of Oroya fever, or that of verruga. As the protocols show, however, they manifested only slight constitutional reaction to the inoculations. The erythrocytes were invaded by the organisms to a small extent only, and there was little or no anemia.⁵ The skin lesions, while of the characteristic verruga type, remained localized at or near the sites of introduction of the microorganisms; there was no spontaneous generalized skin eruption such as is observed in human verruga, and in rare instances in *rhesus* monkeys.

A young female chimpanzee (*Pan leucoprymnus*), said to be about 4 years old, was inoculated on Jan. 29, 1926, with the suspension of a nodule excised* from the eyebrow of *M. rhesus* 3¹ on the same day, and also with cultures, grown on leptospira medium, representing the second generation from the original human blood and the first generation from the blood of *M. rhesus* 1.¹ The suspension was inoculated intradermally into the right eyebrow and by scarification on the right side of the abdomen, while on the left eyebrow and left side of the abdomen a mixture of the suspension with cultures was introduced part intradermally and part subcutaneously. The quantities injected were 0.2 to 0.3 cc. 5 cc. of the mixture were injected subcutaneously into the right lower portion of the abdomen, above the inguinal region, the site of inoculation being carefully massaged after the injection.

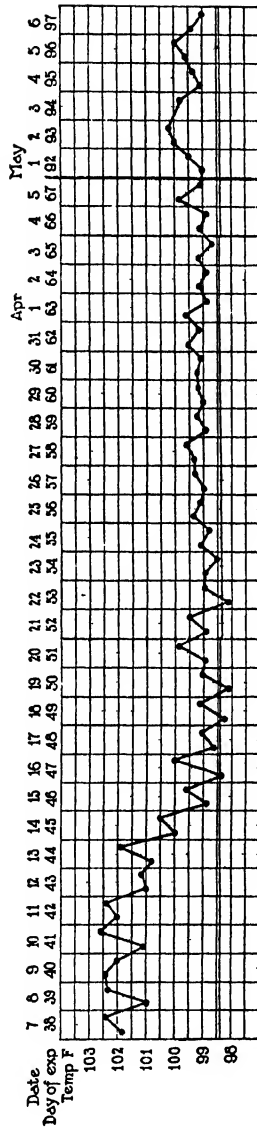
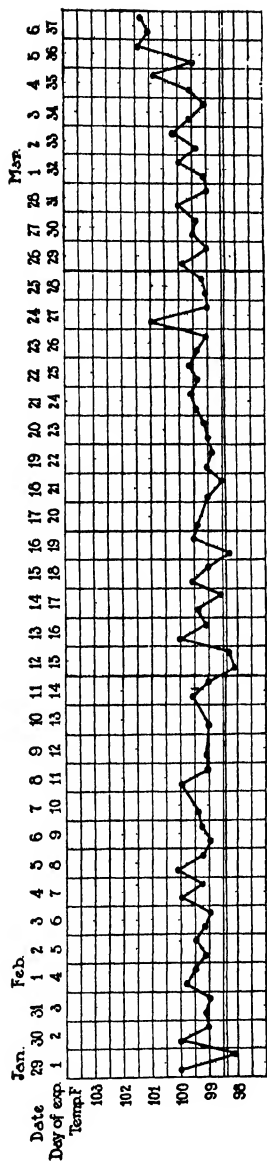
The normal temperature of the chimpanzee, as shown by daily records covering a period of several weeks, varied between 98.6°F. and 99.6°; occasionally the afternoon (4 p.m.) temperature was as high as 100°. Blood counts made before inoculation on Jan. 29 gave the following results: Erythrocytes 5,520,000, leuco-

³ Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

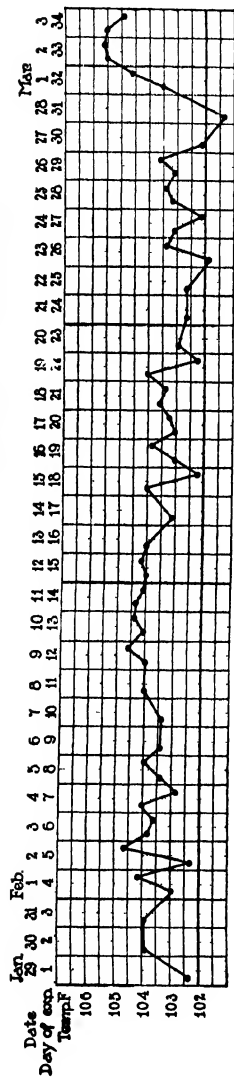
⁴ Landsteiner, K., and Miller, C. P., *J. Exp. Med.*, 1925, xlii, 853.

⁵ The hematological studies were by Dr. J. H. Bauer.

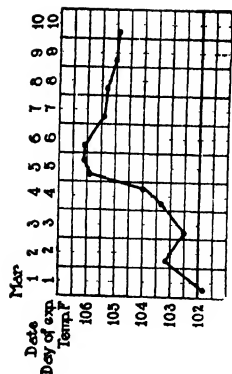
* All operations were performed under ether anesthesia.



TEXT-FIG. 1. Chimpanzee.



TEXT-FIG. 2. *M. rhesus* 14.



TEXT-FIG. 3. *M. rhesus* 21.

All became attached. The animal had been having irregular fever (104°F. or slightly above) for the past 4 weeks, and the unintentional infection with spotted fever by tick feeding escaped attention for a time. When the animal died on Mar. 4, however, it became evident that death was due to spotted fever, and that *M. rhesus* 21 (Text-fig. 3) and the chimpanzee had received on Mar. 1 Rocky Mountain spotted fever virus as well as *Bartonella bacilliformis*. *M. rhesus* 21 died of typical spotted fever within 10 days (4 days incubation, 6 days of high fever, 106°F. during the first 2 days). Notwithstanding the presence of the spotted fever infection, the blood of Monkeys 14 and 21 both yielded cultures of *Bartonella bacilliformis*, that of *M. rhesus* 21 in a dilution of 1:100,000. Injections into guinea pigs of the blood and of suspensions of the nodular tissue from Monkey 14 induced typical fatal spotted fever.

Spotted Fever in the Chimpanzee.—As the chart shows (Text-fig. 1), the spotted fever infection (Mar. 1 to 15) gave rise in the chimpanzee to a very different temperature curve from that of the pure infection with *Bartonella bacilliformis* (Jan. 29 to Mar. 1). After an incubation period of 5 days the temperature rose to 101°F., then gradually to 102.5°F., then fell to a slightly subnormal level, and remained there for several days. The fever was relatively mild, as compared with that usually observed in spotted fever infection in man, *Macacus rhesus* monkeys, and guinea pigs; in these animals spotted fever gives rise to a temperature of 105–106° lasting 7 to 10 days. The chimpanzee had become inactive on the 5th day, was disinclined to move about, depressed, and indifferent to food. This condition continued for 5 days (until Mar. 9). By this time the accident had become known through the death of *M. rhesus* 14, and 20 cc. of pooled spotted fever antiserum from hyperimmunized rabbits were given intravenously to the chimpanzee and also to the control *rhesus* (No. 21). In the *rhesus* the injection had no effect; the animal died the following day. In the chimpanzee the character of the fever before and after the administration of the serum appeared to be the same, and the recognition of curative effect is difficult unless the short duration of the fever is assumed to have been due to the effect of the serum. In *rhesus* monkeys and guinea pigs, however, the serum has not been known to influence the course of fever unless given during the incubation period.⁷ At all events, the chimpanzee recovered very rapidly and probably without aid of the antiserum.

During the period of spotted fever infection, when the animal was very ill, all the nodules, including the reddened skin lesions, became paler and smaller,

⁷ Noguchi, H., *J. Exp. Med.*, 1923, xxxvii, 383.

and the face of the animal was cyanotic, though the color had returned within a week after the time of injection of the serum.

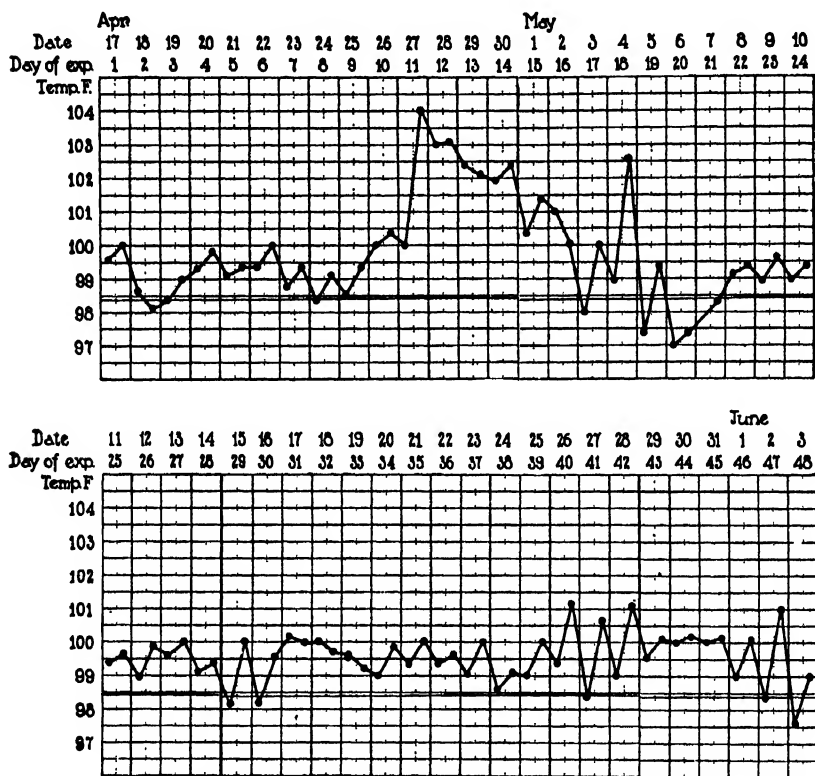
Mar. 1, 1 p.m. Intravenous injection of 1.5 cc. of a mixture of suspension of nodular tissue of *M. rhesus* 14 (removed Feb. 27 and Mar. 1) and cultures of *Bartonella bacilliformis*. *Mar. 5*. The animal ate very little and was inactive. The nodules on the eyebrows were still large (Fig. 12). *Mar. 6*. Temperature 101°F. *Mar. 7*. Temperature 102° a.m., 102.5° p.m. *Mar. 8*. Temperature 101° a.m., 102.5° p.m. Animal inactive, apathetic, appetite poor. The abdominal wounds, where the lesions had been removed, had partly opened, and the surrounding tissues were edematous. *Mar. 9*. Temperature 102.5° a.m., 102° p.m. The animal was very ill. The nodules were pale and cyanotic. Erythrocytes 4,856,000, hemoglobin 80 per cent. 20 cc. spotted fever antiserum given intravenously at 12 noon.

Mar. 10. Temperature 101° a.m., 102.5° p.m. The color had returned to the nodules, and the animal was somewhat more active. Appetite improved. *Mar. 11*. Temperature 102° a.m., 102.4° p.m. The animal was more active, and the color of the face and nodules more nearly normal. *Mar. 12*. Temperature 101° a.m., 101.2° p.m. Eyelids edematous. Erythrocytes 4,288,000, hemoglobin 65 per cent. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:100. *Mar. 13*. Temperature 101° a.m., 101.8° p.m. The monkey had removed some of the stitches from the wounds caused by excision of the lesions; the wounds were healing satisfactorily, however. *Mar. 14*. Temperature 100.5° a.m., 100.5° p.m. *Mar. 15*. Temperature 99° a.m., 99.5° p.m. Nodules smaller and paler, not cyanotic. Erythrocytes 3,488,000, hemoglobin 60 per cent. Blood culture negative. One of the inguinal glands on the left side was removed for examination. The suspension yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10. *Mar. 17*. Temperature 98.4° a.m., 99° p.m. The animal was very active. Erythrocytes 4,344,000, hemoglobin 65 per cent. *Mar. 20*. Temperature 99.5° a.m., 100° p.m. The remaining nodules had diminished considerably in size and the overlying skin was of normal color. *Mar. 25 to 31*. Temperature normal. The skin lesions had healed and showed slight brownish pigmentation. The site of the abdominal nodule was practically flat, and the induration had disappeared. The nodules on the eyebrows were reddish but small (3×4 mm.).

Apr. 2. The nodules on the eyebrows were much smaller. The lymph nodes had also decreased in size considerably. *Apr. 6*. Blood culture negative. Erythrocytes 4,456,000, hemoglobin 75 per cent. *May 1*. The nodules had practically disappeared. Neither the blood nor the lymph yielded cultures of *Bartonella bacilliformis*. *May 3*. Erythrocytes 5,800,000, hemoglobin 85 per cent. The animal had completely recovered.

The Behavior of Bartonella bacilliformis in the Ourang-Utan.

The response of the ourang-utan to the inoculation of *Bartonella bacilliformis* was similar to that of the chimpanzee, but in this instance there was a moderate febrile reaction (Text-fig. 4) which began 10 days after inoculation and lasted about a week. On the day before the onset of fever *Bartonella bacilliformis* was demonstrable



They had disappeared, however, by Aug. 15 (4 months after inoculation).

The ourang-utan (*Pongo pygmaeus*), a young female (probably 2 to 3 years old), was inoculated Apr. 17, 1926. The right eyebrow was scarified and smeared with a piece of nodule just excised from *M. rhesus* 23 and was intradermally inoculated, on the inner aspect, with 0.2 to 0.3 cc. of a suspension of the same tissue. 0.2 cc. of the suspension was injected intradermally into the shaved skin of the right upper abdominal wall, and about 1 cc. was smeared over a scarified area on the right lower side of the abdomen. The left side of the abdomen was inoculated similarly at two sites with a mixture of cultures derived from the blood of *M. rhesus* 7 and *M. rhesus* 18. The mixture included cultures grown on leptospira medium for 10 days and blood agar cultures 6 days old which contained a large number of motile organisms.

The animal received intravenously a mixture containing 6 cc. of the cultures, 1 cc. of the suspension of nodular tissue of *M. rhesus* 23, and 3 cc. of citrated blood from each of two monkeys, *M. rhesus* 11 and *M. rhesus* 24. Two attempts to introduce the syringe needle into the basilic vein of the left arm having failed, the material was inoculated into the corresponding vein of the right arm. A small amount of material from the syringe had, however, been unintentionally introduced subcutaneously into the left arm, and two large subcutaneous nodules later developed at the sites of the attempted intravenous injections.

A blood count made on Apr. 2, 1926, had shown 5,940,000 erythrocytes per c.mm., 12,200 leucocytes, and 85 per cent hemoglobin. The normal temperature of the animal had been taken daily at 11 a.m. and at 4 p.m. for a period of several weeks. It varied from 98.6°F. to 99.6° and was rarely as high as 100° in the afternoon.

Blood taken on Apr. 26, 9 days after inoculation, yielded growth of *Bartonella bacilliformis* in a dilution of 1:100, and a few cells containing the organism were found in film preparations. The erythrocytes at this time numbered 5,392,000, and the hemoglobin was 70 per cent. For the 10 days following inoculation the temperature remained within normal limits, but on the afternoon of Apr. 27 it rose to 104°, and on Apr. 28 it was 103° both morning and afternoon.

Apr. 29. Temperature 102.4° a.m., 102.2° p.m. Appetite poor. Lymph glands enlarged. Slight induration at site of needle puncture on right arm. Erythrocytes 4,880,000, hemoglobin 75 per cent. Apr. 30. Temperature 102° a.m., 102.4° p.m. Animal quiet and apathetic. May 1. Temperature 100.4° a.m., 101.4° p.m. May 2. Temperature 101° a.m., 100° p.m. May 3. Temperature 98° a.m., 100° p.m. Diarrhea. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:10. Erythrocytes 4,808,000, hemoglobin 80 per cent. Slight induration at sites of intradermal injection on each eyebrow.

May 4. Temperature 99° a.m., 102.6° p.m. May 5. Temperature 97.4° a.m., 99.4° p.m. May 6. Temperature 97° a.m., 97.4° p.m. Distinct nodules

at sites of inoculation on eyebrows and areas of induration at the sites of the attempted intravenous injections on the left arm.

From this time on the temperature varied within normal limits. On May 11 the erythrocytes numbered 5,632,000, and the hemoglobin was 80 per cent. Blood taken on May 17 yielded cultures of *Bartonella bacilliformis*. The induration at the site of intradermal inoculation on the right eyebrow had disappeared by May 13, but the adjacent lines of scarification had become distinctly raised and reddish, and there was a distinct nodule, 5×6 mm., at the site of intradermal inoculation on the left eyebrow. The area of scarification on the lower right side of the abdomen also showed activity, being markedly raised and reddish. May 20. Distinct nodules on left side of abdomen at sites of inoculation of cultures. One of these was excised, and also a piece of skin from the scarified area on the lower right abdomen. Both showed the characteristic structure of lesions induced on the skin by *Bartonella bacilliformis*, and the organisms were demonstrated in sections of the tissue (Figs. 6, 25, and 26).

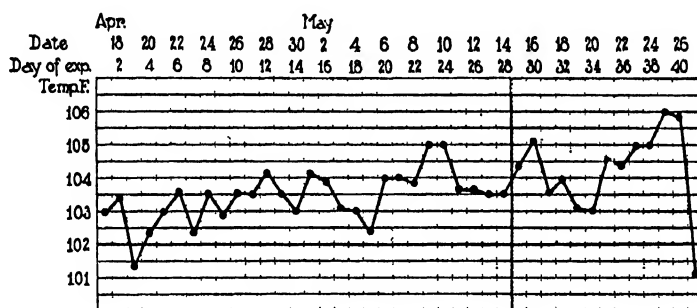
May 23. One of the nodules on the left arm measured 2.5×3 cm., the other 5×5 mm. (Fig. 24). The nodule on the left eyebrow was about 8×9 mm., and there were numerous small reddish papules on the scarified area of the right eyebrow (Fig. 23). All the lesions continued to increase in size until about June 1, when the photographs shown in Figs. 23 and 24 were taken. June 2. Blood culture negative for *Bartonella bacilliformis*. Erythrocytes 5,512,000, hemoglobin 80 per cent, leucocytes 11,600. Polymorphonuclears 33.3 per cent, small lymphocytes 45.25 per cent, large lymphocytes 3.8 per cent, large mononuclears 11.0 per cent, transitionals 1.0 per cent, eosinophils 0.7 per cent, mast cells 4.75 per cent.

The monkey scratched the nodule on the left eyebrow until on June 8 she had succeeded in opening it and on June 10 had removed nearly half of it. The remaining portion was excised on June 10 for examination and culture (Fig. 4). The papules on the right eyebrow had become dry and scaly at this time, and the abdominal lesions had practically disappeared.

In the *rhesus* monkey, inoculated with the same material as the orang-utan to serve as a control, the local reactions were less severe than those often observed in this species, but there was a tendency to generalization of the skin lesions, and the systemic reaction was very severe. Within 7 days after inoculation the sites of intradermal injection on the eyebrows were definitely indurated, and there was a slight spontaneous eruption on the abdomen. The temperature rose on the 11th day after inoculation, and there was continuous high temperature (104 – 106°F.) after the 20th day (Text-fig. 5). The hemoglobin at the time of death, 40 days after inoculation, was 15 per cent, and the erythrocytes numbered 1,176,000.

M. rhesus 37 was injected intravenously (saphenous vein) on Apr. 17, 1926, with the same mixture of cultures and nodular suspension which the ourang-utan had received. The right eyebrow was inoculated intradermally and by scarification with the suspension of the nodule of *M. rhesus* 23, the left in the same way with the mixture used for intravenous injection. The shaved skin of the lower right side of the abdomen was inoculated by scarification with the nodule suspension of *M. rhesus* 23, the lower left side with a suspension of nodular tissue of *M. rhesus* 18. The latter suspension was also inoculated by scarification on two sites on the upper abdomen.

Apr. 23. Slight induration at the sites of intradermal inoculation on the eyebrows. *Apr. 24.* Indurations on the eyebrows definite; slight spontaneous eruption on abdomen. *Apr. 28.* Temperature 104.2°F. Distinct nodule at site of intravenous injection on the right leg and red papules at all four sites of scarification on abdomen. *Apr. 29.* Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:10. Erythrocytes 5,180,000, hemoglobin 70 per cent.



TEXT-FIG. 5. *M. rhesus* 37.

May 1. Temperature 104.2°. *May 3.* Nodule on left eyebrow 2 × 3 mm. *May 7.* Lines of scarification on right eyebrow slightly raised. Nodules on left eyebrow and on leg increased in size. *May 12.* Erythrocytes 5,536,000, hemoglobin 75 per cent. *May 19.* Blood culture negative.

From May 19 to May 24 this animal was used for the feeding of nineteen ticks (*Dermacentor variabilis*). On May 20 there was a spontaneous papular eruption on the abdomen. The nodules on the eyebrows had not increased in size, but the one on the right leg was 4 × 5 mm. On May 26, when the temperature was 105.8° the animal was used for the feeding of ten bedbugs, which were removed the following morning. *May 27.* Temperature 101°; animal very ill, cyanotic. *May 28.* Died.

Autopsy.—Profuse hemorrhage on the abdominal wall and in the omentum but no free blood in the peritoneal cavity. Peritoneal fluid yellowish, slightly turbid, contained a few red cells. Intestines smooth and glistening, no evidence of peritonitis. Lungs whitish gray, extremely anemic, but otherwise normal.

No fluid in the pericardium, no evidence of pericarditis. Heart muscle heavily congested and very hemorrhagic. Liver very pale and soft but showed no gross pathological changes. Spleen normal in size but very dark in color and much harder than normal. Kidneys normal in appearance. There were a few dark bluish enlarged lymph nodes in the mesenterium and one in the large intestine, but otherwise the intestinal tract appeared normal. Axillary and inguinal lymph glands very slightly enlarged. One small nodule on the left eyebrow and another larger one on the back of the left leg. Both on section showed the presence of *Bartonella bacilliformis*.

Erythrocytes 1,176,000, leucocytes 3,200, hemoglobin 15 per cent. Polymorphonuclear leucocytes 50.7 per cent, small lymphocytes 31.1 per cent, large lymphocytes 3.2 per cent, large mononuclears 11.8 per cent, transitionals 1.9 per cent, eosinophils 0.8 per cent, mast cells (basophils) 1.25 per cent, megalo-blasts 1.0 per cent.

The red cells stained very irregularly, a large number of them remaining practically unstained, while about 25 per cent showed marked polychromatophilia. There were a large number of microcytes and a few macrocytes, mostly of irregular shape. No nucleated red cells were found. A few red cells contained *Bartonella bacilliformis*. There were a few threads of fibrin in the smears but no platelets.

The majority of the polymorphonuclear leucocytes had become so changed that the cytoplasm had practically disappeared. The nucleus was still intact. Most of the lymphocytes were extremely small, about half the size of the red cells.

SUMMARY.

The inoculation of a chimpanzee with cultures and a passage strain of *Bartonella bacilliformis* induced local reactions which, while definite and characteristic, progressed less rapidly and were much less striking than those in the control *rhesus* monkey. *Bartonella bacilliformis* was demonstrated in the blood corpuscles with difficulty, and the fever was slight compared with the high and persistent fever of the *rhesus* monkey. In both the swelling of the lymph glands was an early symptom and constantly present. Definite anemia developed in the chimpanzee only after accidental infection with Rocky Mountain spotted fever and may have been due to either one or both infections, though it disappeared when the blood had become negative by culture for *Bartonella bacilliformis* and the local lesions had disappeared. Incidentally, the chimpanzee was found in this one instance to be less susceptible to the spotted fever than *Macacus rhesus* and guinea pigs.

In the ourang-utan, also, *Bartonella bacilliformis* induced a mild systemic and local infection. A rise of temperature occurred 10 days after inoculation, and fever continued for a week, though it was decidedly less severe than that in the control *rhesus*. The lesions induced by scarification were less definite than those which arose at the sites of intradermal inoculation. *Bartonella bacilliformis* was recovered from the blood on the 9th and on the 16th days after inoculation and from nodules excised on the 33rd and 53rd days. A few erythrocytes containing the organism were demonstrated in stained smears, but prolonged search was required to find them.

The symptoms and lesions observed in the chimpanzee and ourang-utan as a result of infection with *Bartonella bacilliformis* are far milder than those of *rhesus* monkeys and show less resemblance to human Oroya fever or verruga.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. *Bartonella bacilliformis* in the erythrocytes of *Macacus rhesus*. Selected cells from film preparations of the blood of *M. rhesus* 25 and *M. rhesus* 30. Giemsa's stain. $\times 1,750$.

FIG. 2. *Bartonella bacilliformis* in the erythrocytes of Case S. A. 15, from which the organism was isolated. Giemsa's stain. $\times 1,750$.

FIG. 3. The nodule excised from the abdomen of the chimpanzee 31 days after inoculation. Natural size and color.

FIG. 4. The nodule excised from the eyebrow of the ourang-utan 53 days after inoculation. Natural size and color.

FIG. 5. *Bartonella bacilliformis* in a section of the chimpanzee nodule shown in Fig. 3. Giemsa's stain. $\times 1,750$.

FIG. 6. *Bartonella bacilliformis* in a section of a nodule of the ourang-utan, excised 33 days after inoculation. Giemsa's stain. $\times 1,750$.

FIG. 7. *Bartonella bacilliformis* in a section of a spontaneous nodule of *M. rhesus* 25 excised 23 days after inoculation. For comparison. Giemsa's stain. $\times 1,750$.

PLATE 26.

FIG. 8. Appearance of the nodules on the eyebrows of the chimpanzee 27 days after inoculation. $1/2$ natural size.

FIG. 9. The skin lesion on the right side of the abdomen of the chimpanzee as it appeared 25 days after inoculation.

FIG. 10. Section of the lesion shown in Fig. 9, which was excised 32 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 11. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 12. The appearance of the nodules on the eyebrow of the chimpanzee 31 days after inoculation.

FIG. 13. The abdominal nodule of the chimpanzee as it appeared 25 days after inoculation.

FIG. 14. Section of the nodule shown in Fig. 13, which was excised 32 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 15. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

PLATE 27.

FIG. 16. Appearance of the nodules on the eyebrows of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 17. The skin lesions on the abdomen of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 18. Section of one of the skin lesions shown in Fig. 17, excised 23 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 19. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 20. The abdominal nodule of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 21. Section of the nodule shown in Fig. 20, which was excised 28 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 22. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

PLATE 28.

FIG. 23. Appearance of the skin lesions on the eyebrows of the ourang-utan 43 days after inoculation. $2/5$ natural size.

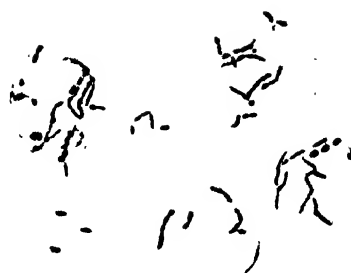
FIG. 24. The large subcutaneous nodules which arose on the left arm of the ourang-utan at the site of unsuccessful attempts to enter the basilic vein at the time of inoculation. Photograph taken 43 days after inoculation. $2/5$ natural size.

FIG. 25. Section of the abdominal nodule of the ourang-utan, excised 40 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 26. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.



Blood of *M. rhesus*.



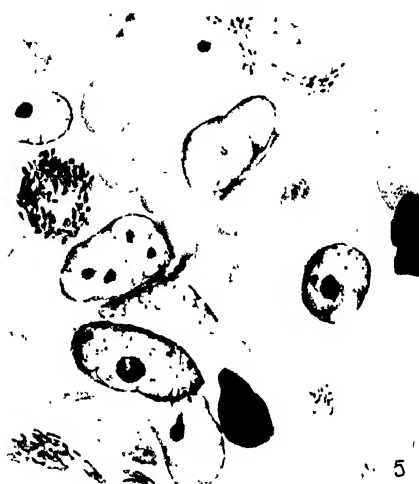
Human blood.



Abdominal nodule, chimpanzee



Eyebrow nodule, orang-utan



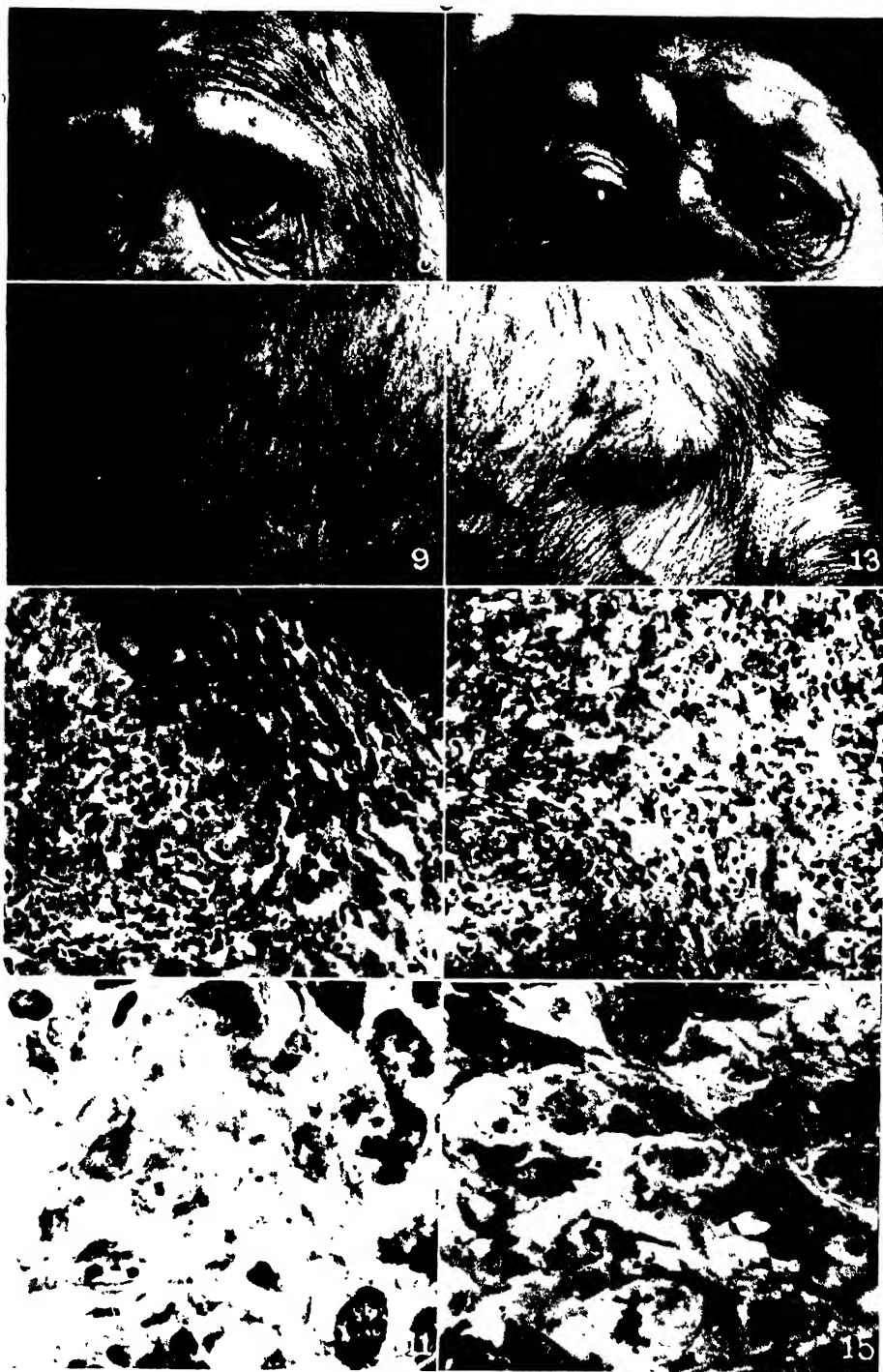
Bartonella in chimpanzee nodule

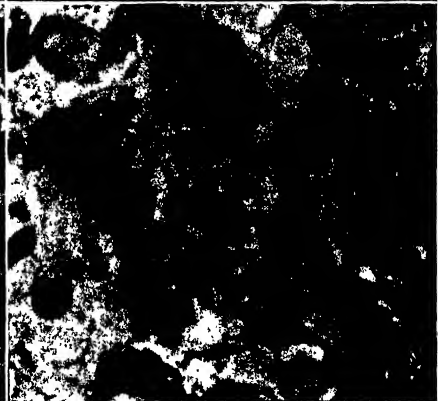
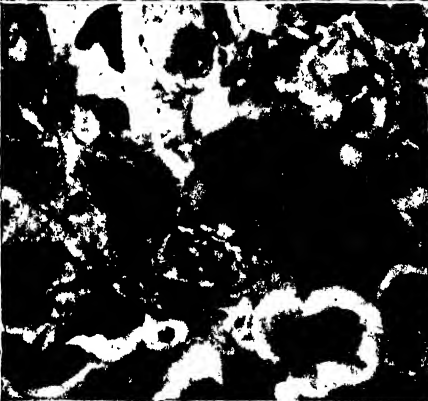
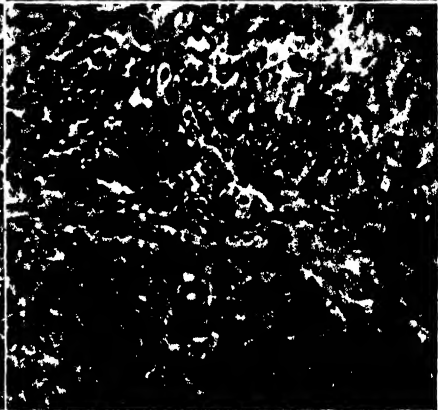
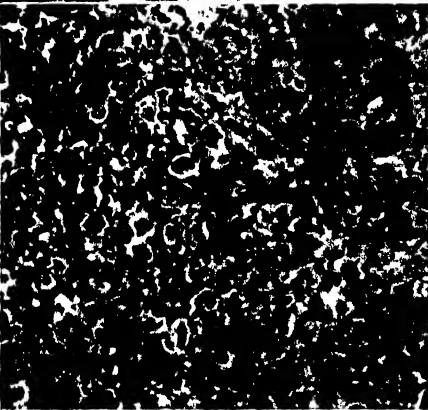
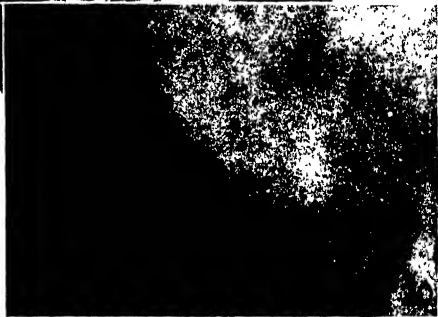


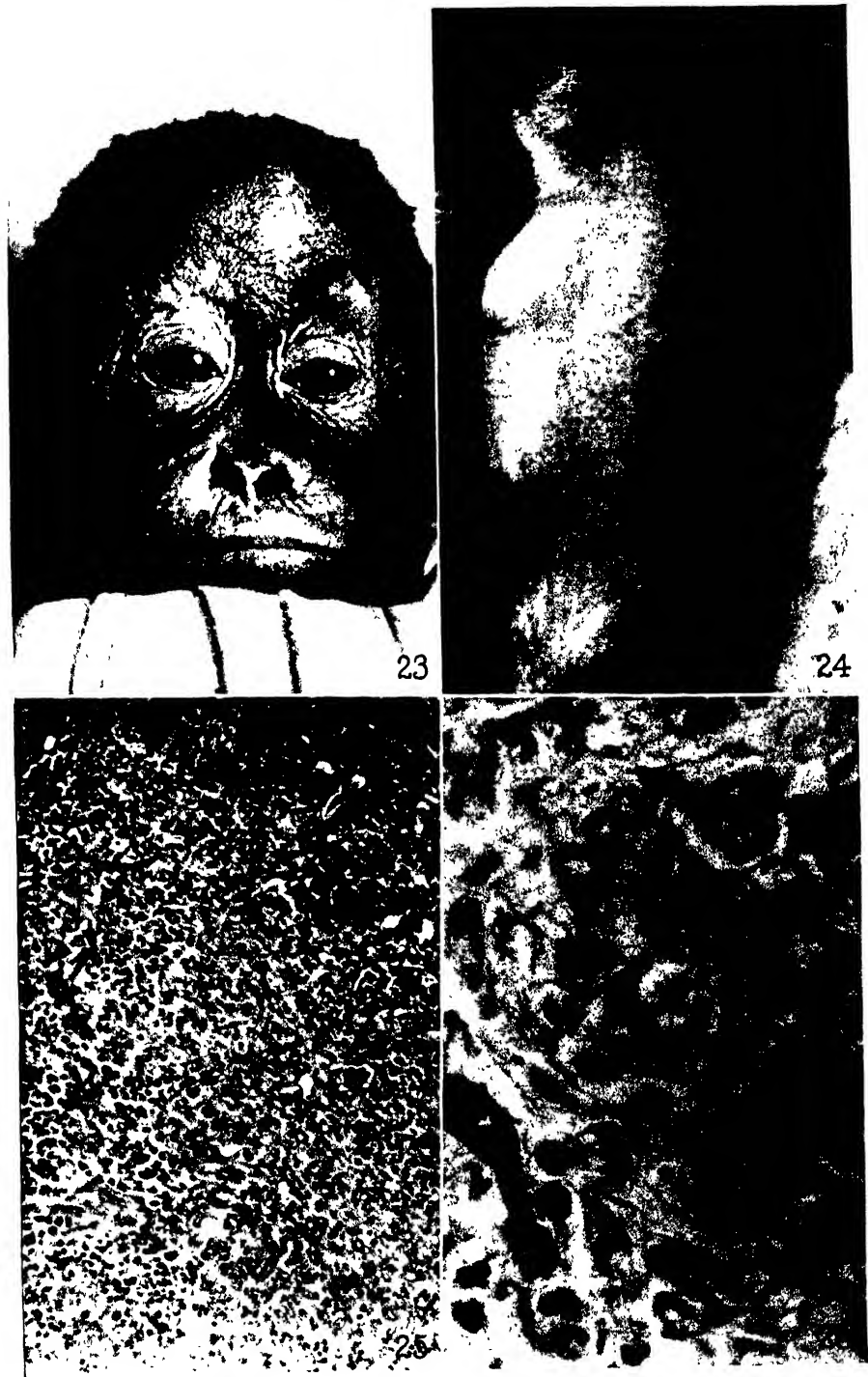
Bartonella in orang-utan nodule.



Bartonella in nodule of *M. rhesus* 25.







(Noguchi: Etiology of Oroya fever. IV)

ETIOLOGY OF OROYA FEVER.

V. THE EXPERIMENTAL TRANSMISSION OF *BARTONELLA BACILLIFORMIS* BY TICKS (*DERMACENTOR ANDERSONI*).

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The geographical distribution of Oroya fever, which occurs within certain limited areas of Peru, has suggested to many students of disease that infection is carried by some biting insect or arthropod, and Castellani and Chalmers¹ note particularly the analogous localization of the tick-borne disease, Rocky Mountain spotted fever. Entomological investigation in relation to Oroya fever has not so far implicated any particular insect, with the possible exception of *Phlebotomus verrucarum* Townsend,² and experimental work has been hampered by the lack of animals susceptible to infection with *Bartonella bacilliformis* and methods of cultivating the organism. These obstacles have recently been overcome,^{3,4} however, and the presence of *Bartonella bacilliformis* in insects can now be definitely determined both by cultural methods and by infection experiments. There is considerable evidence,³⁻⁵ moreover, that verruga peruviana is also caused by *Bartonella bacilliformis*, as had long been suspected because of the similar geographical distribution of the two conditions and their frequent association in the same individuals.

It was not possible at the moment to undertake an investigation of insects from the infected districts, but an indirect method of approach to the problem suggested itself, that of determining whether

¹ Castellani, A., and Chalmers, A. J., A manual of tropical medicine, London, 3rd edition, 1919.

² Townsend, C. H. T., *J. Am. Med. Assn.*, 1913, lxi, 1717.

³ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

⁴ Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

⁵ Noguchi, H., and Hercelles, O., *Science*, 1926, lxiv, 121.

some well known carrier of infectious agents—such, for example, as the tick which transmits Rocky Mountain spotted fever,⁶ tularemia,⁷ and a filterable virus pathogenic for guinea pigs⁸—could also act as a vector of *Bartonella bacilliformis* under experimental conditions. Success in transmitting the parasite under such circumstances, while not solving the problem of the actual vector, would nevertheless furnish a starting point for the investigation of the question.

For the specimens of ticks (*Dermacentor andersoni*) used in these experiments, I am indebted to Dr. R. R. Parker, of the United States Public Health Service, Hamilton, Montana. The ticks were first allowed to feed on normal guinea pigs to determine whether or not they were free from the spotted fever virus. If the guinea pigs escaped infection, the ticks were then placed on the shaved abdominal skin of monkeys (*Macacus rhesus*) in various stages of infection with *Bartonella bacilliformis* and allowed to feed for periods varying from 24 hours to 6 days. Tests of infectiveness were made by feeding the ticks on normal *rhesus* monkeys either immediately following their removal from the infected animal or after an interval of 12 to 14 days.

Two series of experiments were made. In the first, the periods of tick feeding were relatively short—24 hours to 4 days, and several days were allowed to elapse between the removal of the ticks from the infected animals and the test feedings. Infection not being obtained under these conditions, in the second series the period of infective feeding was lengthened to 5 days, the ticks were transferred to normal animals immediately after their removal from the infected ones, and 6 days were allowed for the test feeding. Under these circumstances the ticks proved infective.

Experiment 1 (Negative).

Feb. 1, 1926. Thirty ticks were placed on three infected monkeys (Nos. 7, 12, and 14), ten on each animal, and allowed to remain attached for 1 to 4 days.

⁶ Ricketts, H. T., *J. Infect. Dis.*, 1907, iv, 141.

⁷ Francis, E., *J. Am. Med. Assn.*, 1925, lxxxiv, 1243.

⁸ Noguchi, H., *J. Exp. Med.*, 1926, xliv, 1.

M. rhesus 7^a had been inoculated intravenously and intradermally, on Dec. 21, 1925 (41 days previously), with blood and suspensions of nodular tissue from Monkeys 2, 3, and 4. *Bartonella bacilliformis* was present in the blood 4 days after injection and on several occasions during the following month. The local lesions were well developed by Jan. 19 and were excised* on that date. From Jan. 27 to Feb. 1 fever had been continuous, fluctuating between 104° and 105°F. The ticks were left attached for 24 hours (Feb. 1 to 2). Fever continued until Feb. 9. The blood had yielded cultures of *Bartonella bacilliformis* previously in dilutions as high as 1:10,000,000, but it developed subsequently that the titer had fallen to 1:10 at the time of the tick feeding.

M. rhesus 12 had been inoculated intravenously and intradermally on Jan. 19, 1926, with first generation cultures, derived from the blood of Monkeys 4, 6, and 7, and grown for 14 days on leptospira medium. The blood was positive by culture on Jan. 25 and on Feb. 3 (6 and 15 days after inoculation). The intradermal injections gave rise to small nodules, which, however, never progressed to typical lesions but gradually receded within a month to mere traces of induration. The temperature was continuously high (104°) from Jan. 30 to Feb. 5. The ticks were allowed to feed 72 hours (Feb. 1 to 4).

M. rhesus 14^a had been inoculated on Jan. 29, 1926, with a suspension of nodular tissue from *M. rhesus* 3 and with cultures of *Bartonella bacilliformis* derived from human and monkey blood, the material having been introduced intradermally, subcutaneously, and by scarification. The temperature rose after 24 hours, and the animal remained febrile (104–104.6°F.) for 4 days. The ticks were allowed to feed on the animal Feb. 1 and 2. Blood taken on Feb. 8 yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10 only. The local and general symptoms progressed steadily until on Feb. 23 the blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000, the nodules on the eyebrows were very large and protruding, and those on the abdomen measured 1.5 × 3 cm. It is evident that the ticks were placed on the animal too early in the course of the infection.

Feb. 16, 1926. Three ticks of each lot of ten were placed on the shaved abdominal skin of *M. rhesus* 19, and two of each lot were dissected and suspensions of the viscera inoculated intradermally into the shaved skin of the eyebrows and abdomen of *M. rhesus* 20. The suspensions were tested also by culture. No infection could be demonstrated in the ticks, and the cultures were negative for *Bartonella bacilliformis*.

M. rhesus 19. Nine ticks, of which three had been fed on *M. rhesus* 7, three on *M. rhesus* 12, and three on *M. rhesus* 14, were placed on the shaved skin of the

* All operations were performed under ether anesthesia.

abdomen on Feb. 16, 1925, and allowed to remain 4 days. All became attached. The animal showed no signs of infection—no enlargement of the lymph nodes, fever, or skin lesions. Because of prolapse of the rectum, the animal was killed by etherization on Mar. 6, 18 days from the time of tick feeding. Nothing abnormal was found at autopsy, and cultures of heart blood, bone marrow, spleen, lymph nodes, liver, kidneys, testis, and lungs were negative for *Bartonella bacilliformis*.

M. rhesus 20. Inoculated Feb. 16, 1926, intradermally into the shaved skin of eyebrows and abdomen with the mixed suspensions of the viscera of ticks fed on Monkeys 7, 12, and 14. There were small indurations at the sites of intradermal inoculation on the eyebrows after 10 days, but they disappeared within 3 weeks. The abdominal skin showed no reaction to the injections. The temperature rose to 104° or higher (104.6°) on several occasions (Feb. 19, 21, and 24, Mar. 7 and 11). No swelling of the lymph nodes was noted during 42 days of observation.

Experiment 2 (Positive).

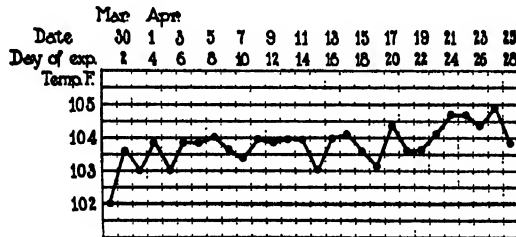
Four ticks were allowed to feed on each of two infected monkeys (*M. rhesus* 18 and *M. rhesus* 23) for 5 days, Mar. 24 to 29, 1926.

M. rhesus 18^a had been inoculated on Feb. 15, 1926, intradermally and by scarification on the shaved eyebrow with a suspension of nodular tissue from *M. rhesus* 5. On Mar. 5 and 18 the blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100,000. The nodules on the eyebrows became extremely large. During the time of tick feeding (Mar. 24 to 29) the temperature fluctuated daily between 104° and 105°. On Mar. 27 the blood was positive in a dilution of 1:10.

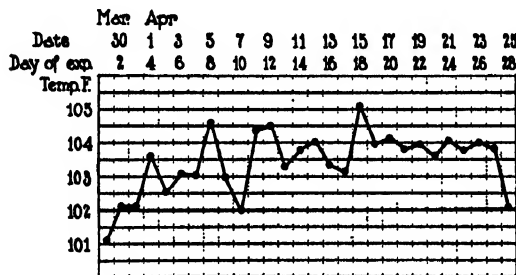
M. rhesus 23 had been inoculated Mar. 9, 1926, intravenously and by scarification with fourth generation cultures derived from *M. rhesus* 7, and intradermally with a suspension of the nodule excised from *M. rhesus* 18 on that date. The local inoculations induced in time very striking nodules and indurations, and blood taken on Mar. 22 yielded growth of *Bartonella bacilliformis* in a dilution of 1:100,000. The temperature was 104°F. on Mar. 24, when the ticks were placed on the abdominal skin. All were moderately engorged when removed on Mar. 29. The course of infection in this animal subsequently became very severe both locally and systemically. There was a prolonged period of fever, and 2 weeks later spontaneous miliary eruptions appeared on the face. The animal was sacrificed May 1, 42 days after inoculation.

On removal from the infected animals, each lot of ticks was immediately placed on a normal *rhesus* monkey and allowed to feed for a period of 6 days. After the feeding the ticks were dissected, and mixed suspensions of the viscera were inoculated into another normal

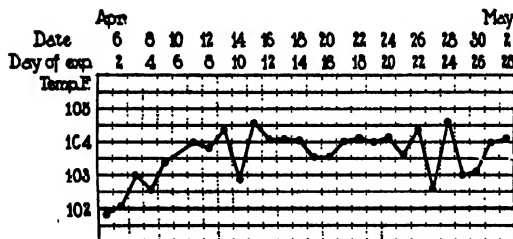
monkey. Definite, though mild, infection was induced in all the animals, and *Bartonella bacilliformis* was recovered in culture from the blood and lymph nodes. The suspensions of the tick viscera also yielded cultures of the organism.



TEXT-FIG. 1. *M. rhesus* 26.



TEXT-FIG. 2. *M. rhesus* 27.



TEXT-FIG. 3. *M. rhesus* 28.

M. rhesus 26. Mar. 29, 1926. Four ticks which had been allowed to feed for 5 days on *M. rhesus* 18 were placed on the shaved skin of the abdomen and left there for 6 full days. On removal, two of the four were found dead, the other two were well engorged. The animal began to show irregular mild fever (Text-fig. 1) 11 days from the time the feeding began, and the lymph glands in

the inguinal and axillary regions on both sides were somewhat swollen. On Apr. 14 one of the inguinal lymph nodes was excised for examination. A suspension of the tissue yielded cultures of *Bartonella bacilliformis*. The temperature was 104–105°F. from Apr. 21 to 25, and blood taken on Apr. 24 yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10. The enlargement of the lymph glands has steadily progressed up to the time of writing (May 30, 1925.) No skin lesions have developed.

M. rhesus 27. Mar. 29, 1926. Four ticks which had been allowed to feed for 5 days on *M. rhesus* 23 were placed on the shaved skin of the abdomen and allowed to feed for 6 full days. All the ticks became attached and were moderately engorged at the time of removal. The reaction in this animal was much the same as that in the foregoing monkey. Cultures made with a suspension of one of the inguinal lymph nodes excised on Apr. 14 and with blood withdrawn on Apr. 24 were positive in dilutions of 1:10. The animal has shown irregular febrile reactions (Text-fig. 2), but no skin lesions have developed up to the time of writing.

M. rhesus 28 (Text-fig. 3). Apr. 5, 1926, inoculated intradermally on the left eyebrow with a suspension in 1 cc. saline of two ticks fed on *M. rhesus* 26 and on the right eyebrow with a suspension in 2 cc. saline of four ticks fed on *M. rhesus* 27. Six-tenths of a mixture of the two suspensions was injected intravenously. The course of events in this animal was very similar to that in Monkeys 26 and 27. Small nodules appeared at the sites of intradermal injection on the eyebrows about 8 days after inoculation, but they did not progress further. The lymph node removed from the inguinal region on Apr. 14 and blood withdrawn on Apr. 24 both yielded cultures of *Bartonella bacilliformis*. A few papular eruptions had appeared on May 19. The skin became yellowish in color about a month after inoculation and has remained so up to the time of writing.

SUMMARY.

Experiments are reported in which *Bartonella bacilliformis* was transmitted from infected to normal *rhesus* monkeys by the bite of the tick, *Dermacentor andersoni*. A long period of feeding, both on the infected animal and on the normal animal subjected to infection, was required in order to secure positive results. The infection transmitted by the ticks was mild, but definite, as shown by the recovery of *Bartonella bacilliformis* from the lymph nodes and blood.

COMPARATIVE STUDIES ON VESICULAR STOMATITIS AND FOOT-AND-MOUTH DISEASE.

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INTRODUCTION.

The similarity in the clinical picture of vesicular stomatitis and of foot-and-mouth disease in cattle is at times strikingly close. The determination of a distinction between the two should be important from the points of view of epidemiology and control. Hence, during the course of the Commission's studies on the virus of foot-and-mouth disease, an investigation was made on the comparison of one disease with the other. It was thought advisable to present at this time a preliminary article on the work. The more comprehensive and detailed experiments will be given in the complete report to be published later.

In this communication, we shall describe comparative studies on the filterability of the two viruses; their action in guinea pigs, cattle, swine, and horses; the immunity induced, and the results of cross-immunity tests with the different animals.

* The Commission appointed by the U. S. Department of Agriculture for the study of foot-and-mouth disease. We wish to express our thanks for the invaluable assistance of Dr. Louis Boëz of the Institut.

Experimental Vesicular Stomatitis in Guinea Pigs.

Waldmann and Pape¹ succeeded in producing experimental foot-and-mouth disease quite regularly in guinea pigs. Their results were amply confirmed by others,² including the British³ and American Commissions. The transfer of this virus to guinea pigs is now regarded as an established fact.

Our first experiment in a comparative study of vesicular stomatitis and foot-and-mouth disease began, therefore, with an attempt to transfer the virus of the former to guinea pigs. Portions of the tongue of a horse showing lesions were received November 18, 1925 from Washington, D. C., in 50 per cent glycerol. This was the only strain of vesicular stomatitis virus available for our work. A physiological salt solution suspension of this virus was applied to the scarified posterior pads of three guinea pigs on November 21, 1925. On November 22 all three showed inoculation, or primary, vesicles.* Generalization of the disease, as manifested by secondary-vesicle formations on the pads of the front limbs of one guinea pig, appeared on November 29. The other two failed to exhibit secondary lesions. Cotton⁴ has also recently reported the transmission of vesicular stomatitis to guinea pigs.

Secondary Lesions.—In general, the virus of vesicular stomatitis induced in guinea pigs lesions similar to those yielded by that of foot-and-mouth disease. In our experience with these viruses it was found that as a rule the vesicular stomatitis virus was slower in inducing the primary and secondary vesicles. The primary vesicles appeared from the 30th to the 48th hour, and the secondary usually from the 72nd to the 96th hour after inoculation. But this is not a definite distinction between the viruses. For, infrequently, there may be a delay in the appearance of the lesions of foot-and-mouth disease and, occasionally, as after the first inoculation with vesicular stomatitis virus, the primary vesicles may appear within the first 24 hours.

Of 250 guinea pigs inoculated with vesicular stomatitis virus, one-

* The term "primary" vesicle as employed in this paper refers to the vesicle at the site of inoculation and the term "secondary" vesicle refers to generalization of the disease, made manifest by the appearance of vesicles at points other than the site of inoculation.

half developed secondary vesicles, while the remainder showed only primary lesions. With foot-and-mouth disease virus, on the other hand, the development of the secondary vesicles is the rule. Nevertheless there are some strains of foot-and-mouth disease virus which may also fail to produce secondary lesions especially in early, guinea-pig passages, in a certain, though small, number of cases. While there is a difference between the time of the appearance of the lesions and the number of cases in which secondary vesicles develop, this offers no definite points of differentiation of the viruses.

Table I indicates, furthermore, that the number of guinea-pig passages has no appreciable effect on the incidence of secondary lesions.

TABLE I.

The Duration of Guinea-Pig Passage and Incidence of Secondary Vesicles.

Vesicle development	1st to 10th passage	11th to 20th passage	21st to 30th passage
Guinea pigs showing primary but no secondary vesicles (+P-S).....	87	27	11
Guinea pigs showing both primary and secondary vesicles (+P+S).....	92	25	8

Site of Secondary Lesions.—Of all the guinea pigs inoculated by local application of virus of vesicular stomatitis to the skin (injured by scarification or puncture with a fine knife or needle) or by intracutaneous injection, only one animal exhibited a vesicle on the tongue. But this is also not a difference between the viruses, since a number of guinea pigs inoculated with some strains of foot-and-mouth disease virus also fail to develop vesicles on the tongue. However, when larger doses of vesicular stomatitis virus were employed, and these were injected either intramuscularly or intradermally in hairy areas, no vesicles developed at the site of inoculation, but, in a considerable number of cases, lesions were noted on the tongue.

Thus of fifteen guinea pigs inoculated intradermally in the abdominal region, six, and of fifteen inoculated intramuscularly, three, exhibited lesions on the tongue. The erosions following the rupture of the vesicle in these cases were more severe and healed much more slowly than those which occurred after inoculation of the foot-and-

mouth disease virus in the pads of the feet. One animal of these series, yielded as a result of intramuscular inoculation, vesicles on two feet as well as on the tongue; a single animal showed on intradermal injection lesions on the feet only; the remaining nineteen failed to yield visible manifestations of the disease.

From the foregoing, it will be observed that in the guinea pig there is little, if any, difference in the effects of foot-and-mouth disease virus or that of vesicular stomatitis.

Cross-Immunity Tests of Vesicular Stomatitis and Foot-and-Mouth Disease in Guinea Pigs.—We then attempted a study of the immunity reactions of these two viruses. In Table II will be found a summary of results:

Test 1 shows that eighteen guinea pigs which had recovered from vesicular stomatitis failed to show lesions after inoculation with vesicular stomatitis virus; and of thirty-one guinea pigs which had recovered from one or more types of foot-and-mouth disease, thirty were susceptible to the virus of vesicular stomatitis.

Test 2 demonstrates that fourteen guinea pigs, recovered from vesicular stomatitis, exhibited primary and secondary vesicles after inoculation with Type-A foot-and-mouth disease virus. Moreover, six control animals, recovered from the Type-A virus, revealed no secondary lesions, but in three only mild primary vesicles after reinoculation with the same Type-A virus.

Test 3 reveals that of eleven vesicular stomatitis convalescents, all showed primary and secondary vesicles after injection with Type-O foot-and-mouth disease virus. Of the twelve recovered from Type-O foot-and-mouth disease virus, ten were solidly immune against this strain, and two showed mild primary but no secondary lesions.*

* Immunity in foot-and-mouth disease may be local and manifest itself by the prevention of primary-vesicle formation at the site of inoculation, or it may be general and prevent the development of secondary lesions. The local immunity is the first to disappear. Thus guinea pigs may still possess immunity against a strain of foot-and-mouth disease virus even though primary inoculation vesicles do occur, provided such guinea pigs are protected from secondary vesicles against a virus which constantly produces secondary lesions. The guinea pigs recorded +P -S with the viruses used are considered immune to that virus. More detailed discussion of immunity will be found in our later report.

From these experiments it will be noted that vesicular stomatitis virus can very readily and regularly be distinguished from the two types of foot-and-mouth disease virus by cross-immunity tests in guinea pigs. Therefore this test should aid in differentiating the two diseases.

Cross-Immunity Tests with Vesicular Stomatitis and Foot-and-Mouth Disease Immune Sera.—The serum of an animal, collected a short time after recovery from foot-and-mouth disease, contains virucidal antibodies which can be demonstrated in the guinea pig. This is interpreted by the protective action of the serum against the virus when both are injected locally into guinea pigs at the same time. The serum in certain quantities protects against the generalization of the disease; that is, no secondary lesions are induced.

The serums of animals recovered from vesicular stomatitis possesses this property also. It was found that serum collected from guinea pigs, 12 to 15 days after inoculation, in quantities as small as 0.1 cc., prevented the generalization of the disease (smaller quantities were not employed). Such vesicular stomatitis immune serum, however, had no protective action against the formation of secondary lesions in foot-and-mouth disease, when doses as large as 3 cc. were employed (larger doses were not used), and, conversely, foot-and-mouth disease immune serum in amounts of 3 cc. had no protective action against the formation of secondary lesions in vesicular stomatitis.*

This summary reveals that the immune serum produced by one virus fails to protect against the other, thus adding another aid in differentiating the viruses.

Experimental Vesicular Stomatitis in Cattle.

Our attention was then directed to a study of the lesions produced in large animals by both these viruses.

Vesicular stomatitis, like foot-and-mouth disease, is characterized by the formation of vesicles on the mucous membrane of the mouth

* The subject of serum-immunity tests upon guinea pigs is discussed at length in our forthcoming report. Without this discussion, figures which we could present on cross-immunity serum tests would not be complete. We have therefore omitted tables on this phase of the work at this time and merely present conclusions deduced from these experiments.

TABLE II.
Results of Cross-Immunity Tests of Vesicular Stomatitis and Foot-and-Mouth Disease in Guinea Pigs.

Test	Guinea pigs in test	First injection			Second injection		
		Virus used*	Result†	Days before second exposure	Virus used	Date	Result
1	18	Vesicular stomatitis	11+P+S 7+P-S	22-25			18 Negative
	12	Foot-and-mouth O-1	12+P+S	21-54			8+P+S 4+P-S
	11	Foot-and-mouth A†	6+P+S 5+P-S	35-54			8+P+S 3+P-S
	3	Foot-and-mouth O-1	3+P+S	92-94		Jan. 15, 1926	2+P+S
		Foot-and-mouth A	3+P+S	36			1+P-S
	3	Foot-and-mouth A	2+P+S 1+P-S	76-81			1+P+S
		Foot-and-mouth O-1	3+P+S	51			2+P-S
		Foot-and-mouth A	2+P+S	74-76			1+P+S
	2	Foot-and-mouth O-2	2+P+S	46			1 Negative
	14	Vesicular stomatitis	9+P+S 5+P-S	31-61			14+P+S
2	6	Foot-and-mouth A	4+P+S 2+P-S	43-56	Foot-and-mouth A	Jan. 21, 1926	3 M§P-S 3 Negative

3	11	Vesicular stomatitis	9+P+S 2+P-S	39-66	Foot-and-mouth O-1	Jan. 25, 1926	11+P+S
	12	Foot-and-mouth O-1	12+P+S	29-84			10 Negative 2 MP-S

* Vallée and Carré⁵ introduced the letters "O" and "A" to designate two types of foot-and-mouth disease viruses. The disease produced by these viruses cannot be distinguished from each other, but one type of virus does not immunize against the other. In these tests one vesicular stomatitis virus, two Type-O and one Type-A foot-and-mouth disease viruses were used.

† +P-S = Primary vesicles but no secondary lesions. +P +S = Primary and secondary vesicles.

‡ In the early guinea-pig passages with foot-and-mouth disease virus Type-A secondary lesions were not induced regularly.

§ M = Mouth.

TABLE III.
Results of Inoculations of Cattle with Vesicular Stomatitis Virus.

Source of virus	Date	Method of injection of or exposure to the virus								Remarks
		Local	Result	Intra-muscular	Result	Intra-venous	Result	Natural	Result	
Horse 1 (vesicle coverings)	2-17-26	4	4 Pos.	4	4 Neg.			6	3 Pos. 3 Neg.*	
Guinea pigs 834 and 835, 837 and 839	3-24-26	2	2 Pos.							
Guinea pigs 821-23, B-75	3-10-26					1	1 Neg.			
Horse 4	3-29-26	2	2 Pos.							
Guinea pigs 849 and 850	3-26-26	1	1 Pos.							
Horse 4	3-29-26	2	2 Pos.							
Heifer 28	3-30-26	1	1 Pos.	2	2 Neg.	7	2 Pos. 5 Neg.			1 positive (foot lesion only)
Guinea pigs 976-981	5-2-26			8	8 Neg.					
Totals		12	12 Pos.	14	14 Neg.	8	6 Neg. 2 Pos.	6	3 Pos. 3 Neg.	

* Record of exposure in the three negative cases:

1. Heifer 8 was placed in same stall with heifer 3 on Feb. 17, 1926. On this day heifer 3 was inoculated locally with vesicular stomatitis virus and showed lesions of this disease, Feb. 19, 1926.
2. Heifer 15 was placed in same stall with heifer 5 on Feb. 17, 1926. On this day heifer 5 was inoculated locally with vesicular stomatitis virus and showed lesions of this disease, Feb. 19, 1926.
3. Bull 18 was placed in same stall with heifer 7 on Feb. 19, 1926. Heifer 7 showed first symptoms of vesicular stomatitis on that day.

which are followed by erosions. Before and during the appearance of the vesicles fever occurs in either condition. In foot-and-mouth disease, as a rule, the lesions are more extensive and more severe. But in vesicular stomatitis the injury may be so extensive, and in foot-and-mouth disease so mild, that a differential diagnosis becomes extremely hazardous. In the inoculated animals this condition was oftentimes strongly evident.

Results of Different Methods of Injection.—Table III shows that forty cattle were exposed to the virus of vesicular stomatitis by one of several modes of injection. Twelve cattle inoculated locally exhibited the experimental disease. The injection in each case was made by applying the virus to scarified areas on the upper gum and tongue. Foot lesions were not found in any of the cases, nor did we observe definite secondary vesicles in the mouth, although extension from the point of injection was often seen.

Of the fourteen cattle inoculated intramuscularly with 1 to 3 cc. of heavy suspensions of virus, none showed definite lesions of vesicular stomatitis. One animal so treated revealed a series of very superficial fullness of epithelium on the tongue, but two guinea pigs injected with material obtained on the day of the appearance of the lesions failed to develop the disease. On the other hand, our experience with intramuscular inoculations with foot-and-mouth disease virus, confirming that of others, indicates that this mode of injection is very effective in inducing the disease in cattle.

Eight cattle were inoculated intravenously with a heavy dose of vesicular stomatitis virus. Six remained unaffected, while two responded with the disease. Of these two, one showed a mild lesion on one foot only, material from which induced vesicular stomatitis in guinea pigs. The other positive case yielded lesions around the nostrils and muzzle. These two animals, due to unavoidable circumstances, were handled by the same attendant in the same stable in adjoining stalls. The location of the lesions on the nostrils and muzzle of the one suggests that the virus may have been transferred by the attendant while holding this animal for examination.

Six animals were exposed by contact to others suffering from the disease. Three contracted the malady and three remained well. As the footnote to Table III indicates, the animals were placed in

contact at the time when the lesions first appeared, or even before this time. In foot-and-mouth disease it has been found necessary to insure exposure during the infective stage, to effect contact at this stage and this probably applies also to vesicular stomatitis.

Hence it may be concluded that although the virus of vesicular stomatitis induces lesions indistinguishable from those of foot-and-mouth disease, a certain difference exists between the effects of the two. Intramuscular or intravenous injection of vesicular stomatitis virus, even when massive doses are used, fails to induce obvious lesions regularly. But the same methods employed with foot-and-mouth disease virus yields as a rule the typical experimental affection.

Immunity in Cattle Following Exposure to Vesicular Stomatitis Virus.—Due to the limited time for our studies, we had no opportunity to observe the duration of immunity in cattle following recovery from the natural or experimental disease. From the data presented in the foregoing pages it is evident that immunity to vesicular stomatitis infection should be based upon the resistance to local inoculations, since the intramuscular or the intravenous methods usually fail to provoke lesions of the disease.

The following is a summary of the results of immunity tests in animals which were exposed by different methods to the active agent of vesicular stomatitis.

Three cattle, recovered from the disease induced by local inoculation, were resistant to a subsequent similar injection.

Six cattle failed to react after intramuscular injection. Subsequently four received a local injection; of these, three failed and one responded with the disease. The second inoculation in the positive case was made only five days after the first. The remaining two cattle in this group were given a second injection intravenously with negative results. Hence, no conclusions could be drawn as to their immunity, for this method may fail to reveal obvious lesions.

Six cattle inoculated intravenously, with negative results, were given a second inoculation locally. All six were resistant.

Six cattle were first exposed to the virus of vesicular stomatitis by contact with infected cattle. Three animals developed the disease. The three unaffected ones were given a second injection intravenously

and therefore yielded no evidence upon which to draw conclusions as to their immunity.

These results indicate that immunity in vesicular stomatitis in cattle is rather easily induced. It is important to note that resistance follows intramuscular or intravenous injection of virus, though no visible lesions are produced.

Cross-Immunity Tests with Vesicular Stomatitis and Foot-and-Mouth Disease in Cattle.—The immunity in cattle following exposure to the virus of vesicular stomatitis has just been demonstrated. Animals recovered from foot-and-mouth disease are also resistant to reinjection with the same type of foot-and-mouth disease virus for varying periods of time. In the following are reported cross-immunity tests between the two viruses.

Twenty-six cattle, which had either recovered from vesicular stomatitis or had been inoculated intravenously or intramuscularly with the active agent of this disease, were reinoculated with foot-and-mouth disease virus. Fifteen of the twenty-six were injected locally on the scarified mucous membrane of the mouth or intramuscularly with either Type-O or Type-A virus. All exhibited experimental foot-and-mouth disease. The remaining eleven were placed in contact with animals having Type-O foot-and-mouth disease. Seven of these developed the disease while four remained apparently normal. Of seven normal animals used as controls in this test, four showed the disease and three were still negative when we were compelled to terminate the experiment.

Nine cattle recovered from one or both types of foot-and-mouth disease were injected with vesicular stomatitis virus. Five were given a local inoculation; all later showed the disease. The other four were placed in contact with vesicular-stomatitis-infected cattle. Two of these developed the disease while the other two remained well.

These tests demonstrate similar results to those obtained in the cross-immunity tests in guinea pigs namely, that animals recovered from vesicular stomatitis are susceptible to either type of foot-and-mouth disease and, conversely, animals recovered from either type of foot-and-mouth disease can be readily infected with the virus of vesicular stomatitis.

Experimental Vesicular Stomatitis in Swine.

Mohler⁶ reported the transference of vesicular stomatitis to three hogs by local inoculation. Our experience confirms this finding, as the following shows: Of eight hogs injected locally or intravenously with virus of vesicular stomatitis, six revealed the disease.

The disease produced by intravenous inoculations was clinically indistinguishable from foot-and-mouth disease in swine. The injected animal shows, after 24 to 48 hours, a marked rise in temperature. In 48 hours, as a rule, distinct vesicles appear on the feet, involving the interdigital space, the coronary band, and the heels, and extending in some instances to the plantar surfaces. In some cases the dewclaws are also involved. The animals become very lame and move about with difficulty, often walking on their knees, as is seen in foot-and-mouth disease. Vesicle formation may also occur on the snout. The animals are quite sick and often do not eat for a long period. Within a few days after the vesicles are first seen, the temperature drops to normal, the feet become less sensitive, and healing gradually takes place. After a few weeks a distinct line of separation between the new and old horn can be seen clearly on the affected feet. These signs and symptoms are identical with those of foot-and-mouth disease.

Following local inoculations, swine show a vesicle at the site of injection and, after its rupture, an erosion with a raw, red base. The lesions appear to be more profound than those of foot-and-mouth disease, induced by local injection. A marked rise of temperature occurs coincident or later than the appearance of the local lesion. In the three animals inoculated in this way no secondary lesions could be seen. In foot-and-mouth disease, however, secondary lesions are noted following local inoculations. The number of hogs employed is, of course, too small to be of any value upon which to establish points of difference between the two diseases.

Two hogs (4 and 7, Table IV) were exposed to natural infection by placing them in contact with others artificially infected and in the same pens. The animals were together from the time the latter were injected. Neither of the two exposed animals exhibited the disease. Hog 4 was resistant to two later inoculations, one intra-

venous and one local, while Hog 7 was proved to be susceptible by reacting to a later local injection of active virus.

Thus it appears that swine are susceptible to vesicular stomatitis and react with lesions similar to those of foot-and-mouth disease.

TABLE IV.

Results of Cross-Immunity Tests in Swine with Foot-and-Mouth Disease and Vesicular Stomatitis.

Hog	Recovered from virus	Reinjected with virus	Method of exposure or injection	Result
1	Foot-and-mouth O and A*	Vesicular stomatitis	Local	+
2	Foot-and-mouth O and A	do	Intravenous	+
3	Foot-and-mouth O and A	do	do	+
4	Foot-and-mouth O and A	do	Natural Intravenous and Local	— — —
5	Foot-and-mouth O and A	do	Intravenous do	? —
6	Foot-and-mouth A	do	do do Local	— — —
7	Foot-and-mouth A	do	Natural Local	— +
8	Foot-and-mouth O and A	do	do	+
9	Foot-and-mouth A	do	Intravenous	+
10	Vesicular stomatitis	Foot-and-mouth O	do	+
11	do	do	do	+

* Two types of foot-and-mouth disease virus, O and A.

Cross-Immunity Tests with Vesicular Stomatitis and Foot-and-Mouth Disease in Swine.—Cross-immunity tests in swine demonstrate, as in similar tests in guinea pigs and cattle, that animals recovered from foot-and-mouth disease were susceptible to infection with vesicular stomatitis and that hogs recovered from vesicular stomatitis were readily infected with Type O of foot-and-mouth disease virus.

Type-A virus was not used. Table IV shows that of eight hogs recovered from one or both types of foot-and-mouth disease and then inoculated locally or intravenously with vesicular stomatitis virus, all revealed clear-cut evidence of the latter disease, excepting Hogs 5 and 6. Hog 5, as a result of an intravenous inoculation, showed a questionable lesion of the disease on one foot only. This animal was, however, resistant to a subsequent intravenous injection. Hog 6 failed to show any evidence of disease following an intravenous inoculation. It was later found resistant to one intravenous and one local inoculation. Hog 4 recovered from foot-and-mouth disease, was exposed by contact to the vesicular stomatitis virus, but failed to show any evidence of disease. It was resistant to later inoculations, two intravenous and one local, of active vesicular stomatitis virus.

Two hogs (10 and 11, Table IV), recovered from vesicular stomatitis, showed foot-and-mouth disease following an intravenous inoculation of the latter virus.

It is to be noted, therefore, that cross-immunity in this species between the two viruses does not exist.

Experimental Vesicular Stomatitis in Horses.

Five horses were inoculated locally with vesicular stomatitis virus as follows: A small portion of the dorsal surface of the tongue and a portion of either the upper gum or inner surface of the lip were scarified. Suspensions of vesicular fluid and epithelial coverings from either guinea pigs or cattle lesions were then applied to these scarified areas. Four of these horses showed severe lesions on the tongue at the site of inoculation. The fifth failed to show evidence of disease at the points of inoculation. It did, however, reveal a temperature of 104.8°F. on the fourth day. Five days after inoculation, this horse was killed while in the agonal stage of meningitis. The cause of the meningitis was not determined.

In general, the appearance of the experimental disease in the horse is as follows: From 36 to 72 hours after injection the tongue shows, at the site of inoculation, blanched, easily-detachable areas which soon fill with clear, straw-colored fluid. These coalesce, forming

a large vesicle containing as much as 5 to 10 cc. of fluid. Within the next 24 hours the vesicle ruptures, leaving a deep, red, raw erosion, which heals very slowly. Definite secondary lesions have not been observed, although we have seen extension of lesions beyond the points of inoculation.

The lesions at the other points of inoculation, such as on the gum and on the lip, are mild, and limited to small vesicle-formations, followed by erosions which heal rapidly. In one of the four cases no definite lesions developed in any other site than on the tongue. In the horse the tongue appears to be a more suitable place for inoculation of vesicular stomatitis virus than other parts of the buccal mucosa. Such was not our experience in cattle. In cattle there seemed to be no marked difference between the severity of the lesions produced on the tongue and those on other portions of the mucous membrane of the mouth.

Insusceptibility of Horses to Foot-and-Mouth Disease Virus.—Six horses were inoculated, locally or intramuscularly, with one or more types of foot-and-mouth disease virus. These inoculations failed in every case to produce any evidence of disease. The literature contains references to rare instances of natural and experimentally-produced cases of foot-and-mouth disease in horses. However, none of these instances can be regarded as clear-cut and unequivocal. The insusceptibility of the horse to foot-and-mouth disease and its sensitiveness to vesicular stomatitis is therefore the important basis in differentiating vesicular stomatitis from foot-and-mouth disease.

Filtration Experiments.

It is generally accepted that the virus of foot-and-mouth disease is filtrable. The members of the commission, in more extensive observations, found that the virus ordinarily carries an electro-positive charge—its iso-electric range centering at $\text{pH} = \text{about } 8$ —and that this condition influences filtration through the commonly-employed electro-negative filters. To summarize the results of the commission's experiments: The foot-and-mouth disease virus, in its electro-positive condition, is filtrable through the Seitz asbestos disc, through Berkefeld V and N candles, and through the various sizes of Chamberland

bougies up to LII. With respect to the LII filter, electro-positive virus failed to traverse this type of Chamberland bougie, but when the charge of the virus was shifted to electro-negative it then passed freely through this same filter.

On the other hand, the virus of vesicular stomatitis was regarded, at the inception of the commission's studies on its filtration in November, 1925, as non-filtrable. Mohler,⁶ in 1918, and Cotton,⁴ in 1926, reported that the virus failed to pass filters which retain bacteria of the ordinary species such as *B. prodigiosus*.

In the following are reported the results of eighteen different filtration tests from thirteen of which an unequivocal conclusion can be drawn that the virus of vesicular stomatitis is also filtrable and that a distinction between this virus and that of foot-and-mouth disease on the basis of filtrability does not exist.

The first test was made, November 30, 1925, with a Berkefeld V filter.

A new Berkefeld filter, Type V, was employed with a negative pressure of 20 cm. Hg. A vigorously-growing culture of *B. prodigiosus* was first filtered, with the result that the material before filtration yielded a profuse growth but the filtrate remained sterile. Then the aspirated lymph from six guinea pigs with experimental vesicular stomatitis was diluted 1:8 in physiological saline at pH = 7.6 and filtered through the same candle. Two guinea pigs were injected intradermally in the posterior pads with the filtrate and four with the unfiltered materials. All six exhibited the typical lesions of vesicular stomatitis within 48 hours.

Thus, in a carefully controlled filtration experiment, it was found that the virus of vesicular stomatitis was filtrable through a Berkefeld V candle. This experiment was repeated under exactly similar conditions except that the virus material was diluted 1:15 in physiological saline at pH = 7.8. In this case four guinea pigs were injected, two with the unfiltered and two with the filtered substance. All showed typical vesicular stomatitis lesions within 48 hours. Another repetition was made with the virus diluted 1:25 in the saline. Here again the two guinea pigs injected with the unfiltered and the two with the filtered material yielded typical experimental vesicular stomatitis within 48 hours. A fourth test was made with the as-

pirated lymph diluted 1:20 in phosphate buffer at pH = 7.5. The other conditions of the experiment remained the same. Three guinea pigs injected with the unfiltered and three with the filtered virus showed the typical vesicles of vesicular stomatitis within 48 hours.

Hence, in four different tests, in which active virus was used from different sources, and diluted from 1:8 to 1:25, either in physiological saline or phosphate buffer at pH = 7.5 to 7.8, filtration was effected through an equal number of new Berkefeld V candles, impervious to a culture of *B. prodigiosus*.

The next experiments concerned the filtrability of the virus through Berkefeld N candles, which are less porous than the V type.

In all, ten different filtration tests were made, employing a new Berkefeld N filter in each instance. These filters were all tested for absence of leakage with *B. prodigiosus* cultures and in every case the unfiltered culture grew profusely but the filtered material remained sterile. A negative pressure of 20 cm. Hg. was used throughout these tests. The sources of the virus were the vesicular fluid or finely ground tissue covering the vesicles from the tongue of a heifer and of four different horses. In addition there was employed the vesicular fluid from the foot lesions of a hog and those of three guinea pigs. The diluents were either physiological saline solution or phosphate buffer at pH = 7.5 to 7.6 and the dilutions of the virus materials in them were from 1:10 to 1:150. In each experiment, as a control, the unfiltered, diluted virus was injected into two guinea pigs which showed, within 48 hours, as a rule, the typical vesicles of vesicular stomatitis. The filtrate was injected intradermally in the posterior pads of from two to three guinea pigs for each test. The results of the inoculation of filtrates are given in Table V.

This series of ten separate filtration tests, made with active virus diluted 1:10 to 1:150, and obtained from different sources, shows that the active agent was also filtrable through Berkefeld N candles which were impermeable to *B. prodigiosus*. In four of the ten experiments, the virus, however, failed to traverse these filters.

Filtration of the virus through a Seitz filter was then attempted. The Manteufel, E. K. model with one asbestos disc, was used at a negative pressure of 20 cm. Hg. The virus comprised the fluid and ground coverings of a vesicle on the tongue of a horse, diluted 1:150

TABLE V.
Results of Inoculation of Filtrates.

Test	Source of material and dilution	Injection of filtrate in guinea pigs
1	Tongue vesicle, horse (1:25)	Positive
2	Foot vesicle, guinea pig (1:10)	Positive
3	Tongue vesicle, horse (1:10)	Negative
4	Foot vesicle, hog (1:15)	Negative
5	Foot vesicle, guinea pig (1:40)	Negative
6	Foot vesicle, guinea pig (1:40)	Negative
7	Tongue vesicle, horse (1:20)	Positive
8	Tongue vesicle, horse (1:150)	Positive
9	Tongue vesicle, heifer (1:20)	Positive
10	Tongue vesicle, heifer (1:20)	Positive

in phosphate buffer at pH = 7.5. Both the unfiltered and filtered material could induce experimental vesicular stomatitis in guinea pigs.

Finally, filtrations were made through Chamberland bougies, sizes L3, L7, and L11,* all of which were proved impermeable to living *B. prodigiosus* cultures.

Single experiments were made with each of the L3, L7, and L11 types of Chamberland filters. No negative or positive pressure was applied, but the virus materials were allowed to drip through the walls for about 1 hour. The virus consisted of the fluid or ground coverings of vesicles on the tongues of two horses with vesicular stomatitis, diluted about 1:20 in phosphate buffer at pH = 7.5 to 7.6.

The unfiltered material from each horse was injected in three guinea pigs, or six in all. These six showed the typical vesicles of vesicular stomatitis within 48 hours after injection.

The filtrate of the L3-type bougie was inoculated into three guinea pigs. One showed primary and secondary vesicles; the other two were apparently unaffected. But a reinoculation of one of the negative guinea pigs with active virus, 15 days later, again revealed no symptoms. Hence lesions may have been present after the first

* An L5-type filter was also employed, but it was found to contain Javelle water (a chlorin compound) with which this filter was cleaned. The negative results in this case, therefore, could not be considered as conclusive.

injection but were probably so slight as to be masked or overlooked. The other negative pig died.

The filtrate of the L7-type bougie was inoculated into three guinea pigs. One exhibited the typical disease which could be transferred to a normal animal, but the second and third were negative. On reinoculation with active virus, 14 days later, one of the latter two was positive, the other negative. It is apparent that here again the symptoms after the first injection may have been overlooked in one of these animals.

The filtrate of the L11-type bougie was injected into three guinea pigs. All remained well and a reinoculation of active virus, after 14 days, induced in these three animals the typical primary and secondary vesicles of vesicular stomatitis.

TABLE VI.
Results of Filtration of the Virus of Vesicular Stomatitis.

Type of filter used	Number of separate filtrations	Number of filtrates containing virus
Berkefeld V	4	4 positive
Berkefeld N	10	6 positive; 4 negative
Seitz	1	1 positive
Chamberland L3	1	1 positive
Chamberland L7	1	1 positive
Chamberland L11	1	1 negative

This experiment demonstrates that the virus can traverse the L3 and L7 types of Chamberland filters but not the L11. In this respect an analogue is to be found in the similar behavior of the foot-and-mouth disease virus—a condition to be discussed later. It is noteworthy that no pressure, positive or negative, was employed in the experiment and that the filters were capable of retaining *B. prodigiosus*.

Table VI summarizes the results of the eighteen separate filtration tests.

To prove the specificity of the virus contained in the vesicular stomatitis filtrates, a cross-immunity test was made in which a Berkefeld N filtrate of the virus was injected into three guinea pigs recovered from vesicular stomatitis; into three having had a Type-A foot-and-mouth disease, and into a like number of animals con-

valescent from Type-O foot-and-mouth disease. The results of this test showed that the filtered vesicular stomatitis virus induced the typical experimental disease in recovered Type-A and Type-O foot-and-mouth disease guinea pigs, as well as in the normal controls, but was without effect in the animals which had been through an attack of vesicular stomatitis.

DISCUSSION.

In the comparative studies of foot-and-mouth disease and vesicular stomatitis, our efforts were directed to find criteria by which one could be differentiated from the other.

Vesicular stomatitis virus produces typical lesions in the guinea pig. In general these lesions are indistinguishable from those of experimental foot-and-mouth disease. It has been pointed out that as a rule the vesicular stomatitis virus requires a longer time to exhibit vesicles than does the foot-and-mouth virus. Furthermore, secondary vesicles were not so constantly present in experimental vesicular stomatitis in guinea pigs. These findings were not constant for the virus of either disease and cannot be relied upon as criteria for differentiating the two diseases.

The cross-immunity tests in guinea pigs, however, indicate definitely that one virus does not immunize against the other. Hence this can be used in supplementing other means of differentiating vesicular stomatitis from foot-and-mouth disease. For example, a supply of guinea pigs recovered from or immunized against vesicular stomatitis may be kept constantly on hand by the Bureau of Animal Industry. Suspected material is inoculated into a number of guinea pigs recovered from vesicular stomatitis and also into normal animals. If the material induces lesions in the normal guinea pigs and not in the recovered animals, it would be tentative proof that the inoculated material contained vesicular stomatitis virus. On the other hand, if both series of guinea pigs became infected, it would indicate that the suspected material contained foot-and-mouth disease virus.

Two types (A and O) of foot-and-mouth disease virus have been briefly referred to. Neither of these viruses immunizes against the other. Whether or not more than one type of vesicular stomatitis

virus exists is not known at present, but such a possibility should, however, be borne in mind when interpreting results of guinea-pig inoculations when vesicular-stomatitis- "immune" animals show lesions. It is not possible under existing conditions in America to have a supply of foot-and-mouth-disease-immune guinea pigs to complete these cross-immunity tests.

The use of foot-and-mouth-disease- and vesicular-stomatitis-convalescent sera in guinea pig tests for differentiating the two diseases also suggests itself.

Cross-immunity experiments with cattle yielded practically the same results as those in guinea pigs. It is apparent, however, that unless cattle recovered from vesicular stomatitis are by chance available, the constant regular supply of immunized guinea pigs is more economical and quite as effective.

Injections of cattle, however, yield differences, which, if carefully considered, may be of considerable aid in differentiating the two diseases. It has been shown that definite lesions of vesicular stomatitis were not observed in cattle inoculated intramuscularly with as much as 1 to 3 cc. of heavy suspensions of vesicular stomatitis virus. On the other hand, with foot-and-mouth disease virus, this mode of inoculation induced typical, pronounced lesions in all eleven cattle injected. Other investigators also report positive results with intramuscular injection of foot-and-mouth disease virus. The intravenous inoculation of vesicular stomatitis virus into seven cattle resulted in one animal showing a definite but mild affection. We did not inoculate foot-and-mouth disease virus into cattle by the intravenous route but others, however, report positive results by this route. Even after local inoculation there is almost always a difference in the behavior of the two viruses. When cattle were inoculated on the tongue or gums with vesicular stomatitis virus, no foot or other secondary lesions were observed. With foot-and-mouth disease virus, local injection as a rule, causes foot and other secondary lesions.

Hogs appear to be at least as susceptible to vesicular stomatitis as to foot-and-mouth disease virus, when inoculated locally or intravenously, and offer no means of differentiating the two diseases.

Cross-immunity tests, however, reveal a sharp difference, but these animals cannot be used with the same advantage as guinea pigs for differential tests.

The susceptibility of the horse to only one of these viruses is the basis for distinguishing the two diseases. While this animal is readily infected with vesicular stomatitis virus, we have been unable to infect it with either type of foot-and-mouth disease virus. The failure to affect the horse by inoculation of foot-and-mouth disease virus, together with field experiences in foot-and-mouth disease in this and other countries, may be accepted as evidence of the resistance of this animal to that disease, in spite of the few indefinite cases to the contrary found in the literature.

It was demonstrated that cattle are readily immunized against vesicular stomatitis by local, intramuscular, or intravenous inoculation of the virus, even though no evidence of the disease is observed. By analogy to the immunity produced in certain other diseases, it was hoped that this strong, easily-produced immunity in vesicular stomatitis would also protect against foot-and-mouth disease infection. A large series of cross-immunity tests proved, however, that cattle recovered from vesicular stomatitis become infected when exposed to foot-and mouth disease virus either by contact, by local, or by intramuscular inoculations of the virus.

In respect to filtration, there is no distinction between the virus of vesicular stomatitis and that of foot-and-mouth disease. Both respond to the test in a fairly parallel manner. It appears that the virus of vesicular stomatitis has a tendency to become adsorbed in the walls of the denser types of filters, as in the case of the Berkefeld N, and the L11 type of the Chamberland. With the foot-and-mouth disease virus this condition also prevails and has been found to be a consequence of its electro-positive charge. No cataphoresis tests were made, however, with the active agent of vesicular stomatitis, so that a definite interpretation of this phenomenon is not possible. The results of the experiments are suggestive, however, if such adsorption occurs as a consequence of electro-positive charge, that the virus of vesicular stomatitis may be of the order of magnitude of that of foot-and-mouth disease.

CONCLUSIONS.

From the foregoing work the following conclusions may be drawn:

1. Vesicular stomatitis virus is readily transferred to guinea pigs. There is little to differentiate this disease from foot-and-mouth disease by a study of the lesions induced. Cross-immunity tests in these animals, however, demonstrate that vesicular stomatitis virus can be distinguished readily and definitely from foot-and-mouth disease virus. This, therefore, offers an aid in differentiating the two diseases.

2. Vesicular stomatitis virus induces lesions in cattle which are at times indistinguishable from those of foot-and-mouth disease. Differences were noted, however, in the response to various methods of inoculation of the two viruses. Divergence in the course of the two diseases was also observed. Immunity produced in cattle by vesicular stomatitis and foot-and-mouth disease is solid for its respective virus, but in the considerable number of cattle inoculated with, or exposed by contact to, the two viruses, no evidence of cross immunity was found.

3. Swine are susceptible to the active agent of vesicular stomatitis; the disease induced is then indistinguishable from foot-and-mouth disease. However, no evidence of cross immunity between the two was discovered.

4. Horses are very sensitive to infection with vesicular stomatitis virus by inoculation on the tongue. On the other hand, these animals are resistant to foot-and-mouth disease when inoculated on the tongue, mucous membrane of the lips, or intramuscularly. The horse can be regarded as the best test animal to differentiate vesicular stomatitis from foot-and-mouth disease.

5. In thirteen out of eighteen carefully controlled filtration experiments it was found that the virus of vesicular stomatitis was filtrable through Berkefeld V and N candles, through Seitz asbestos discs, and through Chamberland bougies, sizes L3 and L7. The virus, similar to that of foot-and-mouth disease, is not filtrable under ordinary conditions through Chamberland L11-type bougies and shows the same tendency to adsorption in the walls of denser electro-negative filters.

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ON GROUP SPECIFIC SUBSTANCES IN HUMAN SPERMATOZOA.

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Experiments have been performed about one year ago with the purpose of examining the presence of group specific substances in human cells other than erythrocytes. We know of a few contributions dealing with this and related subjects. One is a paper on the organs of dogs by von Dungern and Hirschfeld (1), another is by Halpern (2) and a third is a communication by Ashby¹ of which only the title is available.

Of particular interest was the behavior of the sperm cells. If they were found to contain group specific substances these should be present in the cells along with those which determine the heredity of the blood groups. The interesting question then, would arise whether the specifically absorbing substances, namely the isoagglutinogens are themselves involved in the inheritance phenomenon, or not. A similar question presents itself with regard to the species specific substances.

The experiments yielded clear-cut results as to the presence of group specific factors in the sperm cells. We refrained from publication before more information was obtained concerning some other phases of the subject, but a paper by Yamakami, (3) which just appeared, on the group differences of human semen causes us to report our results. Yamakami found that semen and saliva have a specific inhibiting action on the normal human isoagglutinins, and mentions the possible forensic application of the phenomenon.

Our tests differ from those of Yamakami in that his recorded ex-

¹ "A study of the capacity of human tissue to remove isoagglutinins from human tissue," Program of the meeting of the American Association of Immunologists at Washington, May 4, 1925.

periments are carried out with the fluid part of the semen (and saliva) while ours were made with the isolated cells. The only statement made by Yamakami with reference to the sperm cells is as follows:

It is true that this inhibiting power is due partly to the adsorption of agglutinins by spermatozoa, but also it cannot be denied that the fluidal part of the semen, or the semen plasma, has a specific inhibiting property, because the experiments made with the clear or opaque supernatant fluid obtained by centrifugalizing semen give the same results as with the whole semen itself.

A minor difference concerns the technic since Yamakami made use of the inhibiting action of the semen plasma on normal human isoagglutinins; we employed the method of absorption of group specific immune sera obtained from rabbits.

Technic.

For each test at least two and often three specimens belonging to different blood groups were used at the same time. Only a fresh specimen rich in spermatozoa was used in the tests. On allowing it to stand for one hour the heavier prostatic cells and suspended particles settled together with some spermatozoa but the far greater part of the latter was present in the supernatant fluid. This was sucked off and centrifuged at top speed. The supernatant was discarded and the sperms were washed once in saline and resuspended. By counting the sperm cells and bringing the suspensions to about the same degree of turbidity, an equal amount of sediment of each sample was obtained, which corresponded in volume approximately to the sediment from 0.25 cc. of a 2.5 per cent suspension of human blood.

The absorptions were carried out by adding to each sperm sediment in small tubes two drops of a group specific liquid (immune sera abs. with Blood I) and one drop of saline. A control tube contained the group specific liquid and saline. The tubes were thoroughly shaken several times, kept one hour at room temperature and overnight in the ice box, and centrifuged the following morning. The supernatant fluid was tested with the respective blood (II or III) in the following proportions—one drop each of the absorbed fluid, 2.5 per cent suspension of blood and saline. The results were read at the end of one hour.

The results are to be seen from the tables. It appears that a small amount of sperm cells absorb specifically and almost completely the group agglutinins of immune sera. The sperm cells therefore contain substances identical or similar to the isoagglutinable factors A and B of human red blood cells.

Experiments on individual serological differences of sperm possibly bearing upon the subject in question were reported by Dervieux (4) and Süssman (5) who also point out the medico-legal application of their findings. The former described the production of precipitins against human sperm showing a marked individual specificity besides the species specificity (6, 7, 8) and Süssman confirms this part of

TABLE 1.*
Tests with Blood Group II.

Sperm.....	B.B.	W.R.	W.X.	C.H.	I.M.	M.K.	J.J.B.	W.W.	M.Y.	M.Y.
Blood group.....	I	I	I	I	II	II	II	II	III	III
Group II specific liquid absorbed with sperm	++	++	++	++	f. tr.	0	tr.	±	++	++
Group II specific liquid unabsorbed.....	++±	++	++	++	++	++	++	++	++	++

* Table 1 is a composite of 4 individual experiments.

TABLE 2.*
Tests with Blood Group III.

Sperm.....	J.J.B.	W.W.	M.Y.	M.K.	I.M.	M.Y.
Blood group.....	II	II	III	II	II	III
Group III specific liquid (1:20) absorbed with sperm.....	++	++	0	+	+	0
Group III specific liquid (1:20) unabsorbed.....	++			+		

* Table 2 represents two different experiments.

Dervieux's findings. As the number of specimens examined by the authors was limited one may think that the individual differences found are actually group differences. In this case the results would be intimately related to those of Yamakami, considering the findings by Schiff (9) on the existence of group specific precipitable substances in human serum. Süssman and Dervieux seem to conclude that by the use of sperm immune sera more individual differences, apparently

aside from those of the groups, can be demonstrated in the human sperm and perhaps also in the human blood. Since this view would be of far reaching significance with regard to the problem of serological individuality, confirmation of this work on a larger scale is desirable.

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QUANTITATIVE CYTOLOGICAL STUDIES ON THE RENAL TUBULES.

I. THE NUCLEOCYTOPLASMIC RATIO.

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To make our cytological studies even roughly quantitative is a difficult problem, but its desirability is widely recognized. Some cells are naturally more suitable for this purpose than others. The epithelial cells lining the renal tubules of mammals seem to be unusually favorable. They form a continuous layer one cell in thickness throughout the length of the tubule. Their shape is relatively simple and they are not encumbered by irregular processes difficult to estimate quantitatively. From one end of the tubule to the other they are of the same embryological origin. The very interesting and striking differences in form and structure which they present in the several segments of the tubule certainly indicate the existence of division of labor as between sharply limited groups of cells—a fact amply proved by experimental methods.

A vast literature has developed consisting of papers initiated in the hope of using these cells in an attempt to correlate cellular structure and function. Yet the results have been disappointing. There is no unanimity of opinion even on the direction of the passage through them of the constituents of the urine. Histologists, led by Heidenhain, are inclined to suppose that some of the cells actively secrete substances into the urine of the tubules, while Ludwig and his followers believe that they merely adsorb water from the lumen and thus serve to concentrate the fluid passed through the glomeruli.

So much that is almost unavailing has been done that at first sight it might seem that further cytological studies would be superfluous. But, as a matter of fact, some features of the cells have been only very insufficiently examined. Among these may be mentioned: 1) the nucleocytoplasmic ratio, which is the subject of this paper;

2) the mitochondria, and, 3) the Golgi apparatus. To estimate the latter quantitatively will be a difficult task, but with improvements in technique it may not be impossible.

The general plan of these studies is to gather detailed information relating to the structural differences between the cells of the four principal divisions of the urinary tubule, and their distinctive behavior in response to vital dyes, poisons, and under different physiological and pathological conditions. In other words, it is our object to estimate as many factors as possible and to ascertain whether they are independently or dependently variable.

MATERIAL AND METHODS.

The albino rat was selected for this study on the nucleocytoplasmic ratio, because so much has been done, particularly at The Wistar Institute of Anatomy, to establish norms for experimental purposes. Twenty-eight rats were used, of which thirteen were males and fifteen were females. They varied in age from two days to about one year, the body length (nose-anus) ranged from 6.5 cm. to 33.5 cm. and body weight from 8.5 gm. to 340 gm. Donaldson's tables ('26) were used for checking the length, weight, and age of the rats.

Five rats of the same litter were employed for a preliminary and prerequisite study of the influence of various fixatives upon the nucleocytoplasmic ratio. The kidneys of each of the latter were fixed in one or more of the following fluids: 1) Zenker's fluid without acetic acid; 2) with $2\frac{1}{2}$ per cent acetic acid; 3) with 5 per cent acetic acid; 4) with 10 per cent acetic acid; 5) 10 per cent formalin; 6) Regaud's fluid; 7) 95 per cent alcohol or 95 per cent alcohol and ammonia. After completing the usual technical procedures with the material the embedded organs were sectioned transversely at 3μ , stained with Giemsa's stain, and finally mounted in cedar oil (table 1).

For a study of the postnatal volumetric changes in the cells of the renal tubules, the same procedure was followed, with the exception that one kidney of each animal was fixed in Zenker's fluid plus 5 per cent acetic acid, and the remaining organ of each in Regaud's solution to be reserved for the subsequent study of mitochondria.

Several methods for determining the cellular, nuclear, and cyto-

plasmic volumes were tried, with the result that the method described by Jackson ('19) and Godlewski ('21) was finally adopted. Since this method has been adequately described, a brief summary of it, with the necessary modifications for the material under consideration, will be sufficient.

Two or more transverse sections passing approximately through the center of the kidney were made. The cut portions of the various parts of the tubule were then outlined on standard-weight paper by means of the camera lucida at a magnification of approximately 1500. Outlines of parts of the tubules and the nuclei were traced in pen and ink. Care was taken not to change the focus of the microscope while drawing, so that boundaries of tubules and nuclei were outlined in a single optical plane. About 500 nuclei with the surrounding cytoplasm were thus traced for each organ.

The paper, representing the respective portion of each tubule, was then weighed to the milligram on an analytical balance. The nuclear areas were cut out by means of fine curved scissors and their number recorded. The remaining paper which represented the area of the cytoplasm was weighed and the difference between the previously recorded weight and the latter weight gave the weight of the nuclear areas. Because the magnification and thickness of the paper remained constant, the ratio of the nuclear surface to the cytoplasmic surface was readily computed by dividing the weight of the cytoplasmic areas by that of the nuclear areas. This ratio is not only one of surfaces, but is obviously, also, one of volumes. Since each portion of the tubule was outlined in a single optical plane, one is therefore justified in assuming that the result is an approximation of the ratio in terms of volume between nucleus and cytoplasm.

The paper weight of the nuclear areas being known, the actual average nuclear area was then computed by dividing the weight of the paper by the magnification squared, further dividing by the weight of 1 sq.cm. of paper (about 0.012 gm. per sq.cm.), and finally dividing by the number of nuclei. Since this figure represents the average surface area of the nucleus in a single optical plane, it is evident that this is not the average maximum surface. If it is assumed that the nuclei are spherical in form and that the volume of a sphere is equivalent to two-thirds of the volume of its circumscribed cylinder, then

the area of the great circle of the sphere is equal to $2/3\pi r^2$, and $2r$ is then the diameter of the average-sized nucleus. It is further evident that $2\pi r^2$ is the area of the averaged-sized nucleus and that $4/3\pi r^3$ gives the computed volume. For the reason that the cells of the renal tubule are obviously not spherical, the computed diameter, on the basis that they approach a spherical form, would be of but little significance. The cell volume may be computed from the nuclear volume and the nucleocytoplasmic ratio on the assumption that the latter approximates the relation between cytoplasmic and nuclear

TABLE 1.

The Effects of Various Fixations upon the Nucleocytoplasmic Ratio in the Renal Tubule (Five Rats of the Same Litter, Age about Three and One-Half Weeks).

Rat No.	Sex	Fixative	Proximal convoluted tubule	Distal convoluted tubule	Loop of Henle	
					Ascending limb	Descending limb
6	f.	Zenker's	1:5.26	1:6.07	1:5.06	1:3.91
5	m.	Zenker's	1:6.82	1:6.48	1:5.14	1:3.74
5	m.	Zenker's 2½%	1:7.63	1:6.43	1:4.53	1:3.36
7	f.	Zenker's 2½%	1:9.16	1:5.41	1:4.37	1:2.78
7	f.	Zenker's 5%	1:8.71	1:7.79	1:5.07	1:3.56
6	f.	Zenker's 5%	1:8.26	1:7.80	1:5.52	1:4.88
8	f.	Zenker's 10%	1:6.35	1:5.96	1:4.17	1:2.16
7	f.	Formalin 10%	1:6.97	1:5.81	1:5.32	1:3.39
4	m.	Regaud's	1:5.66	1:5.75	1:4.76	1:3.20
7	f.	Alcohol 95%	1:9.21	1:8.08	1:5.17	1:3.70
8	f.	Alcohol + ammonia	1:7.95	1:6.18	1:3.73	1:3.51

volumes. The nuclear volume multiplied by the figure in the ratio representing the cytoplasm gives an approximate figure for the volume of the cytoplasm to which the nuclear volume is added for an estimation of the cell volume.

Direct measurements were made on the nuclei of the tubule cells by means of a filar-wheel micrometer at a magnification of approximately 1500 times. The maximum nuclear diameters of the four parts of the renal tubule were determined for ten rats ranging in age from two days to one year. The average of fifty observations was taken as the maximum nuclear diameter, thus making a total of 200

nuclei measured for each kidney. These measurements were used as a check for the calculated measurements. Only those nuclei in the field under the microscope containing a nucleolus were measured, assuming that the plane of section passed through the center of the nucleus in such instances.

The Nucleocytoplasmic Ratio.

In table 1 are given the ratios for four parts of the tubule as determined for rats of the same litter, but with the use of various fixatives. In the Zenker's series, with and without acetic acid, it is obvious that the ratio changes with the addition of glacial acetic acid. The nuclear and cytoplasmic volumes are not so divergent when material is fixed in Zenker's fluid without the acid, and the same fixative with 10 per cent acetic acid, as when intermediate percentages of the acid are added, the ratios with 10 per cent formalin and Regaud's fixative are similarly less. Ninety-five per cent alcohol, used as a fixative, affects the ratio in a manner comparable to the weaker percentages of acid in Zenker's fluid. With the addition of ammonia to the alcohol, the ratio again is slightly different.

By means of the readings of the volumes of water, alcohol, xylol, and warm paraffin displaced the relative shrinkage for material fixed in Zenker's fixative, and 5 per cent acetic acid was found to be about 17 per cent. The data given in the ensuing tables have not been corrected and are to be regarded as determinations after fixation in Zenker's with 5 per cent acetic acid.

The nucleocytoplasmic ratios in rats ranging in age from two days to one year are given in table 2 and illustrated by means of a histogram in figure 1. It is obvious that the ratios of nuclear and cytoplasmic volumes are different for each of four parts of the tubule, and that the ratio undergoes a change with postnatal development. The convoluted tubules closely resemble one another in the order of magnitude of their ratios; similarly, the limbs of the loop of Henle are comparable. The shifting of the ratio with body growth is one of increased cytoplasmic volume in relation to nuclear volume.

In the proximal convoluted tubule the mean ratio of six determinations on rats of two weeks and less in age is 1:7.6, and the

range is 1:6.45 to 1:8.65. Assuming that the ratio is established by the tenth week, the average of nine determinations on rats ranging in age from ten weeks to one year is approximately 1:9.5 with a range of 1:8.83 to 1:10.73. The increase in the figure, representing the cytoplasmic portion of the ratio, is roughly 2.0.

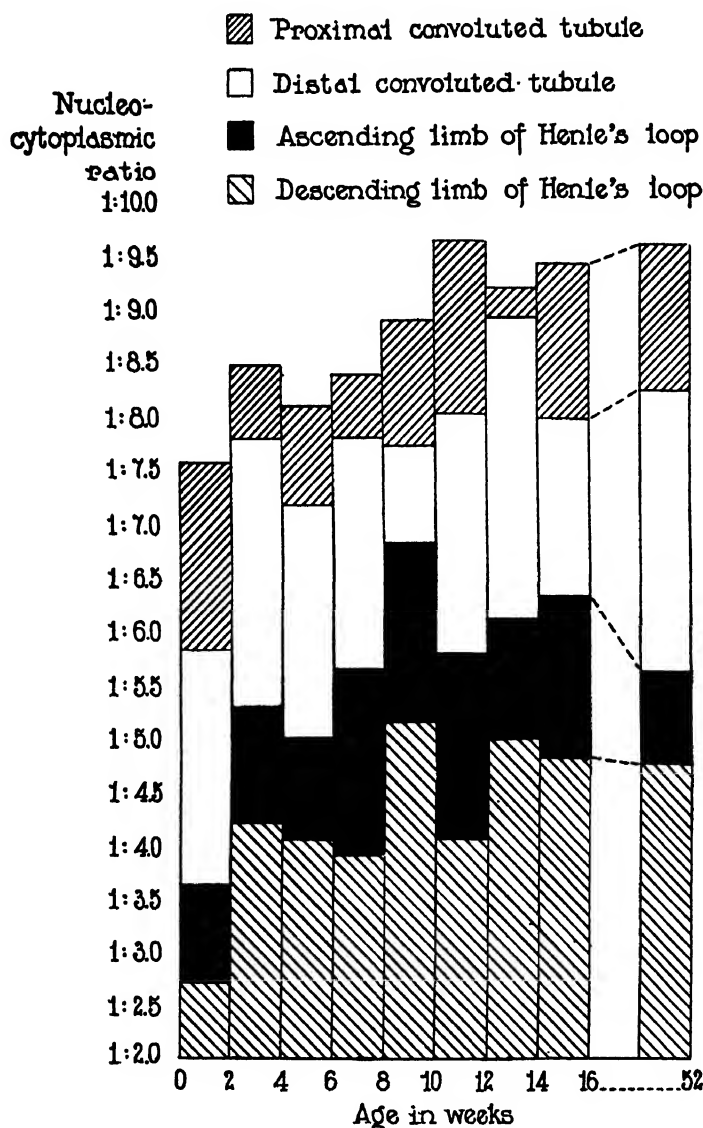
TABLE 2.

Nucleocytoplasmic Ratio (Twenty-Five Cases).

No.	Age in days	Sex	Body length	Body weight	Proximal convoluted tubule	Distal convoluted tubule	Ascending limb of Henle's loop	Descending limb of Henle's loop
			cm.	gm.				
R. 20	2	f.	6.5	8.5	1: 7.81	1: 7.07	1:3.15	1:2.46
R. 22	2	f.	6.5	8.7	1: 7.45	1: 5.47	1:3.30	1:3.23
R. 23	5	m.	6.5	12.4	1: 6.45	1: 4.86	1:2.85	1:2.73
R. 24	5	f.	6.7	12.3	1: 6.68	1: 5.04	1:3.22	1:2.45
R. 25	14	m.	8.8	21.6	1: 8.65	1: 5.86	1:4.06	1:2.74
R. 26	14	f.	8.6	20.4	1: 8.47	1: 6.73	1:5.17	1:2.62
R. 6	26	f.	10.6	35.0	1: 8.26	1: 7.80	1:5.52	1:4.88
R. 7	26	f.	10.8	38.0	1: 8.71	1: 7.79	1:5.07	1:3.56
R. 27	35	m.	14.0	58.0	1: 8.48	1: 7.24	1:5.32	1:4.26
R. 28	35	f.	13.5	57.0	1: 7.74	1: 7.10	1:4.69	1:3.89
R. 29	43	f.	13.7	59.2	1: 8.07	1: 7.18	1:5.09	1:3.33
R. 30	43	m.	13.0	69.0	1: 7.60	1: 7.59	1:5.43	1:3.87
R. 31	50	f.	15.0	95.0	1: 9.33	1: 8.71	1:6.86	1:4.30
R. 32	50	f.	15.0	82.5	1: 9.25	1: 9.10	1:6.47	1:4.39
R. 1	55	m.	15.2	78.0	1: 7.66	1: 6.48	1:4.45	1:3.98
R. 11	69	f.	16.6	105.0	1: 8.89	1: 7.73	1:6.82	1:5.17
R. 3	74	m.	17.5	121.2	1: 9.34	1: 7.64	1:5.96	1:4.66
R. 14	75	m.	17.8	128.0	1: 9.44	1: 6.80	1:6.21	1:3.69
R. 42	82	m.	19.0	150.0	1:10.11	1: 9.62	1:5.21	1:3.86
R. 19	87	f.	18.0	130.5	1: 9.31	1: 7.82	1:6.58	1:4.06
R. 41	95	f.	19.0	150.0	1: 9.09	1:10.04	1:5.64	1:5.94
R. 18	106	m.	16.5	125.3	1: 9.42	1: 7.93	1:6.34	1:4.83
R. 43	125	m.	20.9	215.0	1: 8.83	1: 6.40	1:5.56	1:3.14
R. 44	155	f.	21.0	190.0	1: 9.25	1:10.19	1:5.14	1:5.35
R. 45	1 yr.	m.	23.5	340.0	1:10.73	1: 8.12	1:6.18	1:5.80

The distal convoluted tubule has a mean ratio of 1:5.84 for the six determinations of the youngest group and a range of 1:4.86 to 1:7.07. The values are considerably altered in the interval of the second to the fourth week, and by the tenth week growth changes

apparently do not influence the ratio. The mean of the nine determinations for rats more than ten weeks of age is approximately 1:8.3, with a range of 1:6.40 to 1:10.19. The difference between this ratio



and the one of the group representing the first two weeks of postnatal development is roughly 2.5.

The ratio in the ascending limb of the loop of Henle is about 1:3.6

for the first two weeks of life, and ranges from 1:2.85 to 1:5.17. By the third week, it has shifted to 1:5.0, and by the tenth week no growth changes in the value of the ratio are noticeable. The mean ratio of the older group is 1:6.0 and the range is 1:5.14 to 1:6.58.

The ratios for the descending limb of the loop of Henle in the first two weeks range from 1:2.45 to 1:3.23, and average 1:2.7. From the end of the tenth week to one year, the mean ratio for nine determinations is roughly 1:4.6, with a range of 1:3.14 to 1:5.94.

Estimations of Nuclear and Cell Size.

The estimations of nuclear diameters, volumes, and cell volumes for twenty-five rats, ranging in age from two days to one year, are given in table 3.

Nuclear diameters in the four parts of the tubule are different. The nuclei of the proximal convoluted tubule are about 5.5μ in diameter. For the twenty-five determinations the range is 4.8μ to 5.9μ . In the distal convoluted tubule the mean diameter is approximately 5.6μ , with a range of 5.2μ to 6.2μ . The average nuclear diameter of the ascending limb of the loop is 5.3μ and ranges from 4.8μ to 5.8μ . The determinations for the nuclei of the descending limb of the loop of Henle average 5.0μ , and range from 4.5μ to 5.7μ .

Assuming that the nuclei of the tubule are spherical in form, the volumes may then be computed. The mean of the computed values of nuclear volumes for the proximal convoluted tubule is $89.93 \text{ cu.}\mu$; for the distal convoluted tubule it is $96.24 \text{ cu.}\mu$; for the ascending limb of the loop it is $78.64 \text{ cu.}\mu$, and for the descending limb of the loop it is $68.97 \text{ cu.}\mu$.

In table 4 are given, by way of comparison, the observed and calculated nuclear diameters of four parts of the tubule for ten rats. The observed values are usually slightly less than the computed ones. In the convoluted tubules the means of the calculated values are about 0.3μ more than the observed values. In the remaining portions the difference is still in favor of the calculated values, but it is less marked.

Cell volume, unlike the nuclear volume, shows a distinct tendency toward an increase with growth of the body.

The mean cell volume of the proximal convoluted tubule for the first six determinations in table 3 is $633.87 \text{ cu.}\mu$ and for the last nine

TABLE 3.
Estimations of Nuclear Diameters and Volumes and Cell Volumes.

No.	Age in days	Proximal convoluted tubule			Distal convoluted tubule			Ascending limb of Henle's loop			Descending limb of Henle's loop		
		Nuclear diameter μ	Nuclear volume cm. μ^3	Cell volume cm. μ^3	Nuclear diameter μ	Nuclear volume cm. μ^3	Cell volume cm. μ^3	Nuclear diameter μ	Nuclear volume cm. μ^3	Cell volume cm. μ^3	Nuclear diameter μ	Nuclear volume cm. μ^3	Cell volume cm. μ^3
R. 20	2	5.3	78.84	694.58	5.8	102.20	824.75	5.4	83.37	345.98	5.4	83.37	288.46
R. 22	2	5.8	102.20	694.96	5.8	104.30	674.82	5.4	82.91	356.51	5.2	73.62	311.41
R. 23	5	5.1	68.64	511.36	5.5	85.23	462.81	5.3	77.07	396.71	5.2	74.05	276.21
R. 24	5	5.4	84.29	647.35	5.4	84.29	509.11	4.9	63.89	269.61	4.8	58.63	202.32
R. 25	14	4.8	58.63	565.77	5.4	82.45	565.60	5.3	77.95	394.42	5.0	65.45	244.78
R. 26	14	5.2	72.48	689.23	5.2	71.10	549.60	5.0	67.03	413.58	5.1	68.24	247.02
R. 6	26	5.6	94.94	879.14	5.7	96.97	853.33	5.0	66.24	431.88	4.5	47.71	280.53
R. 7	26	5.5	90.97	833.31	5.6	93.94	825.73	5.3	78.84	478.55	5.7	96.97	442.18
R. 27	35	5.6	90.00	853.20	6.0	113.10	931.94	5.3	79.73	503.89	5.0	66.24	348.42
R. 28	35	5.4	82.91	724.63	5.7	95.95	777.19	5.4	81.54	463.96	5.0	66.24	323.91
R. 29	43	5.6	93.94	852.03	6.2	124.80	1020.86	5.6	90.97	554.00	5.3	76.20	329.94
R. 30	43	5.5	88.55	761.53	5.9	106.40	913.97	5.3	78.84	506.94	5.2	74.05	360.72
R. 31	50	5.4	83.37	861.21	5.9	107.50	1043.82	5.2	74.47	585.33	4.8	57.91	306.92
R. 32	50	5.9	109.70	1124.42	5.8	103.80	1051.08	5.6	91.95	686.86	4.9	60.48	325.98
R. 1	55	5.6	91.95	796.29	5.6	91.95	677.78	5.6	91.95	501.13	4.8	59.37	295.66
R. 11	69	5.7	99.02	979.31	5.7	96.97	846.55	5.3	77.07	602.68	5.0	67.03	413.57
R. 3	74	5.7	97.99	992.63	5.6	92.94	802.00	5.1	68.64	477.73	4.9	61.98	350.80
R. 14	75	5.6	92.94	970.29	5.5	85.23	664.79	5.0	64.67	466.27	5.0	67.03	314.37
R. 42	82	5.3	81.54	905.91	5.5	87.11	925.10	5.6	92.94	577.15	5.2	77.07	374.56
R. 19	87	5.8	102.20	1053.68	5.7	99.02	873.35	5.5	86.17	653.16	5.5	90.00	455.40
R. 41	95	5.7	95.95	968.13	5.7	99.02	1093.18	5.1	70.28	466.66	4.9	61.60	427.50
R. 18	106	5.3	80.63	840.16	5.5	85.23	761.10	4.8	57.19	419.77	5.1	70.28	409.73
R. 43	125	5.7	99.02	973.36	5.6	92.45	650.75	5.8	103.20	676.99	5.2	74.47	308.30
R. 44	155	5.8	102.20	1047.55	6.0	113.10	1265.58	5.5	87.11	534.85	4.9	63.12	400.81
R. 45	1 yr.	5.8	105.40	1236.34	5.5	90.97	829.64	5.1	71.94	516.53	4.9	63.12	429.21
Mean		5.5	89.93		5.6	96.24		5.3	78.64		5.0	68.97	

determinations is 973.27 cu. μ . The estimation of cell volume for the young rats represents approximately 65 per cent of the cell volume of the older group. The average volume of a cell of the distal convoluted tubule for the first two weeks of life is 597.78 cu. μ , which is roughly 68 per cent of the mean of the calculated volumes for rats of more than ten weeks of age. The latter-mentioned figure is 873.94 cu. μ . The average cell volume of the ascending limb of the loop of Henle undergoes a relative increase of about 32 per cent during post-

TABLE 4.

Observed and Calculated Values of Nuclear Diameters for the Various Parts of the Renal Tubule (Fifty Observations for Each Observed Value).

Rat No.	Age in days	Proximal convoluted tubule		Distal convoluted tubule		Ascending limb of loop		Descending limb of loop	
		Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated
		μ	μ	μ	μ	μ	μ	μ	μ
20	2	5.4	5.3	5.5	5.8	5.0	5.4	5.3	5.4
24	5	5.1	5.4	5.2	5.4	5.1	4.9	5.2	4.8
26	14	5.2	5.2	5.3	5.2	5.2	5.0	5.0	5.1
27	35	5.2	5.6	5.4	6.0	5.1	5.3	4.8	5.0
30	43	5.2	5.5	5.4	5.9	5.1	5.3	4.9	5.2
32	50	5.2	5.9	5.4	5.8	5.2	5.6	4.9	4.9
14	75	5.3	5.6	5.3	5.5	5.1	5.0	5.0	5.0
18	106	5.1	5.3	5.0	5.5	5.0	4.8	5.2	5.1
43	125	5.4	5.7	5.3	5.6	5.3	5.8	5.4	5.2
45	1 yr.	5.2	5.8	5.4	5.5	5.3	5.1	5.1	4.9
Mean		5.2	5.5	5.3	5.6	5.1	5.2	5.0	5.0

natal development. The mean cell volume of the youngest group is 364.47 cu. μ , which is increased to 532.12 cu. μ by the end of the tenth week. In the descending limb of the loop of Henle the relative increase is approximately the same as in the other three portions of the tubule. The mean cell volume for the first six determinations is 261.70 cu. μ , while that of the last nine is 385.63 cu. μ . The absolute increase is therefore 123.93 cu. μ and the relative increase approximately 33 per cent.

DISCUSSION.

The determination of the nucleocytoplasmic ratio is a difficult task, for which several methods are available. The morphologist either attempts an approximation of the cell diameters and nuclear diameters by direct measurement and computes each as spheres or determines the ratio by means of the paper-weight method, previously described. Le Breton and Schaeffer ('23) have estimated by chemical methods the ratio of nuclear mass to protoplasmic mass for various stages of development of the body as a whole of the white mouse. They criticise the conclusions as drawn by the morphological methods on the basis that volume and mass have been confused. Both methods should certainly be used where it is possible to do so, but certain organs of the body do not lend themselves to such an analysis, as in the case of the portions of the renal tubules.

With the present status of our knowledge of the urinary tubule, any carefully worked-out information is of value, since it may finally aid in solving the problem which the tubules now present. The fact that both the cell volume and the nucleocytoplasmic ratio vary with the growth of the body is of some significance, though what it means is still unknown. Berezowski ('10) found the size of the intestinal cells to be smaller in younger white mice than in older ones. The ratio of cell size and nuclear size has been correlated with various pathological conditions, such as rejuvenescence and senescence, by different investigators, in other tissues. Thus far, however, the renal tubules have only been studied by simple and rapid methods which do not yield detailed information of this kind.

SUMMARY OF RESULTS.

1. The nucleocytoplasmic ratio varies for the different portions of the renal tubule and shifts during postnatal development. In the proximal convoluted tubule of a mature rat it is roughly 1:9.5; in the distal convoluted tubule it is 1:8.3; in the ascending limb of the loop of Henle it is 1:6.0; in the descending limb of the loop of Henle it is 1:4.6.
2. The nuclear diameter and volume of each of the four parts of the tubule are different, but show no distinct tendency toward a

change with growth in weight. The mean nuclear diameter for the cells of the proximal convoluted tubule is calculated as 5.5μ and the mean nuclear volume as $89.93 \text{ cu.}\mu$; for the distal convoluted tubule the diameter is about 5.6μ and the volume $96.24 \text{ cu.}\mu$; in the ascending limb of the loop of Henle the diameter is 5.3μ and the mean calculated volume is $78.64 \text{ cu.}\mu$; in the descending limb of the loop the diameter is about 5.0μ and the volume $68.97 \text{ cu.}\mu$.

3. The average cell volume for each part of the tubule increases approximately 35 per cent during postnatal growth. The cells of the proximal convoluted tubule and the distal convoluted tubule have a volume of approximately $973 \text{ cu.}\mu$ and $874 \text{ cu.}\mu$, respectively. The cell volumes in the limbs of the loop of Henle are roughly $532 \text{ cu.}\mu$ for the ascending limb and $385 \text{ cu.}\mu$ for the descending limb.

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BACTERIUM PNEUMOSINTES IN CLINICAL INFLUENZA IN NEW YORK CITY IN 1926.

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INTRODUCTION.

Since the first paper in the series by Olitsky and Gates (1) in which the presence of minute filter-passing bodies (*Bacterium pneumosintes*) in cultures from affected rabbits' lungs and from filtered nasopharyngeal washings of influenza patients was reported, a number of investigators in widely separated laboratories have described the isolation, under similar conditions, of anaerobic filter-passing organisms, identified morphologically and sometimes serologically as *Bact. pneumosintes*. Such organisms were "obtained consistently" by Loewe and Zeman (2) in New York, from the filtered nasopharyngeal washings of patients with epidemic influenza, and produced a characteristic clinical and pathological picture (3) when injected into experimental animals. Then Gordon (4) in London reported evidence of the same bacterium in 14 of 20 influenza cases, and filtrates of the bronchial secretion in 3 fatal cases yielded 2 cultures. In 1922 also, Lister (5), in South Africa, obtained 5 cultures of an identical anaerobe in 11 instances in which influenzal washings were cultured within 24 hours of the acute onset, and reported 4 febrile reactions, 1 fever and drop in the leucocyte count, and 1 case of typical influenza among 12 volunteers sprayed with unheated cultures of the filter-passing organism. No reactions occurred among 6 volunteers sprayed with heated cultures. In 1923 Nakajima (6) in Tokio cultivated 2 strains from pharyngeal washings and 1 from the lung tissues of a fatal case of influenza. Seitz (7) in Zurich observed masses of very tiny bodies in the respiratory exudates of influenza patients and grew them for a time in mixed sputum cultures. He regards these bodies as coccoid

rather than bacilloid. Detweiler and Hodge (8) in Toronto grew 3 strains morphologically similar to *Bact. pneumosintes* from filtered influenza material, 2 from lung filtrates of injected animals, 1 from filtered nasopharyngeal washings. Subcultures failed to grow, so identification was not completed. Finally Thomson (9) isolated a minute organism from a case of Engadine fever—a type of influenza endemic in Switzerland—and obtained 2 other strains from influenza patients in England.¹ In all of these studies control experiments with non-influenzal material have been uniformly negative.

Thus the presence of an anaerobic, Gram-negative, filter-passing bacterium, identified as *Bact. pneumosintes*, in the human respiratory tract only in the early hours of an influenzal infection, has been established in many parts of the world. This in itself is but a beginning, however, in determining the relationship of *Bact. pneumosintes* to the clinical disease. A large accumulation of observational and experimental evidence must be sought wherever available, and pieced together as opportunities permit.

The Outbreak of 1926.

When we undertook an investigation of the presence of *Bact. pneumosintes* in clinical influenza in New York City last March (1926), the brief local outbreak proved to be already on the wane. Consequently we had an opportunity to see only a few cases. It was reported that clinically the infections varied considerably in mode of onset, relative prominence of various signs and symptoms, and the blood picture. Yet they would be grouped together with the common designation "influenza" as typified by sudden onset, sometimes with chill, a sharp fever and marked prostration, headache and other pains, absence of profuse coryza, and prolonged depression during convalescence. Sometimes family infections, and more often isolated

¹ While this paper was in press M. W. Hall (*J. Exp. Med.*, 1926, xliv, 539) reported the experimental production of characteristic lung lesions in rabbits and guinea pigs with nasopharyngeal washings from a patient with typical epidemic influenza. From one of his affected animals a culture of *Bact. pneumosintes* was obtained. The presence of this organism in the lungs of experimentally infected animals predisposed them to the pulmonary localization of other bacteria and the production of definite secondary pneumonic lesions.

cases occurred. Compared with the influenza epidemic and its repercussions of 1918 to 1922 this outbreak was characterized in general by the relative mildness of the primary infection and the rarity of secondary complications. Often other signs of typical epidemic influenza, such as flush, photophobia, conjunctivitis, diffuse pharyngitis, and the characteristic leucopenia, were noted, but in the absence of acknowledged proof of the etiologic agent in clinical influenza it would be unwise to draw a close parallel between the infections of this transient outbreak and influenza of the epidemic type. A search for *Bact. pneumosintes* in these sporadic cases thus presented a new field for study and a new problem in its relation to the clinical disease.

In the 10 days that elapsed between our first contact and the failure of available material we obtained nasal and postnasal washings from 9 patients with clinical influenza and from 1 person who developed only a common cold. In several instances the patients had complained of a headache and feeling of depression for a day or two before the acute attack, so that the actual time of invasion is difficult to determine, but the washings were obtained in each instance within 24 hours of the acute onset, marked by fever and prostration in bed. We decided at the outset to follow the established routine in detail and so handled our material as follows:

The patient's nose and throat were washed out with 40 to 50 cc. of sterile, dextrose Ringer's solution. The washings were shaken with beads and divided into two portions. One sample was filtered through a new Berkefeld V candle and used to inoculate Smith-Noguchi medium and *coli* broth (10) under a vaseline seal, and was spread on rabbit blood agar plates for aerobic and anaerobic incubation. The unfiltered nasopharyngeal washings were injected intratracheally by the method of tracheotomy into stock rabbits under light ether anesthesia. The febrile and leucocytic reactions of these rabbits were carefully followed and at autopsy on the 1st or 2nd day, portions of the lung tissue of these animals were ground, and this material, filtered or unfiltered, was inoculated into Smith-Noguchi tubes and on blood agar plates. Fragments were also placed in 50 per cent glycerol, and the rabbits were examined carefully for evidence of concurrent disease.

Rabbit Passages.

Unfiltered washings from 7 of the 9 influenza patients were injected intratracheally into 13 rabbits, usually in amounts of 3 cc. 9 of these

rabbits, representing 6 patients, showed significant reactions, such as fever, a drop in the leucocyte, and especially the monocyte, count, and typical gross and petechial hemorrhages in large, edematous lungs. 6 rabbits injected with whole or filtered lung tissue from 4 of these animals showed similar but less striking effects in the 2nd passage. A 2nd passage apparently failed in 4 other transfers from 2 of the other 5 rabbits.

This series of rabbit injections was carried out under a serious handicap. Our stock of rabbits was low, and the immediate demands of the situation required the use of untested animals. Although only apparently healthy rabbits were chosen for injection, the whole lung tissues of most of them subsequently showed infection with *B. leptosepticus* or *B. bronchisepticus* and cultures of them had to be discarded. In the presence of these concurrent infections a strict interpretation of the reactions of most of these rabbits is not justified. The series was therefore discontinued and our attention turned to the direct cultivation of filter-passing, anaerobic organisms from the nasopharyngeal washings, and from filtered material from the rabbits' lungs.

Cultivation Experiments.

Having in mind that often very sparse growths of filter-passing anaerobes are obtained in early generations, and might easily be missed in the Smith-Noguchi medium, we made at least 2 successive transfers of every primary tube that showed no growth when subplanted on aerobic blood agar plates. The control tubes set up to test the sterility of our media were likewise transferred, to avoid the possibility of false evidence from an extraneous source. Material from each generation was also examined microscopically in stained smears.

This tedious procedure proved to be justified in 6 series of cultures in which minute anaerobic organisms were obtained. 2 of these have been definitely identified as strains of *Bact. pneumosintes*. In the first 2 generations of these *pneumosintes* cultures the growth was so sparse as to escape microscopic detection and no visible colonies developed in subplants on anaerobic blood agar plates. But in the 3rd, 4th, and subsequent generations the typical clouding of the Smith-

Noguchi medium, the microscopic observation of minute, Gram-negative bodies such as have been fully described (10), and the growth on anaerobic blood agar plates of microscopic, discrete, round, convex colonies with an entire edge and a colorless translucency indicated the growth of *Bact. pneumosintes* morphologically identical with the 1918 to 1922 strains.

One strain was obtained from the whole lung tissue of a rabbit injected with unfiltered nasopharyngeal washings, and consequently had not been filtered. Fortunately this rabbit was free from previous lung infection and, as in numerous cases reported by Olitsky and Gates (11), the contaminating bacteria (in this case *S. albus* and diphtheroids) in the unfiltered nasopharyngeal washings were suppressed during the rabbit passage.

The 2nd strain, also obtained through rabbit passages, was derived from the filtered lung tissue of a 2nd passage rabbit, intratracheally injected with whole lung tissue that had stood in 50 per cent glycerol for 31 days. This 1st passage lung tissue had been contaminated with a large Gram-negative bacillus which did not survive glycerolation, so that the lungs of the 2nd rabbit yielded no aerobic growth. The 2nd passage rabbit showed no fever, only a slight leucopenia (a drop in monocytes from 2790 to 2040 cells), and no gross lesions except one small surface hemorrhage in the lungs.

No primary cultures of *Bact. pneumosintes* were obtained in *coli* broth or on anaerobic blood agar plates. These media are only suitable for special purposes with well established strains. This fact emphasizes the importance of the Smith-Noguchi medium for primary cultures, and even in this medium the initial cultivation of *Bact. pneumosintes* is difficult and uncertain. A lesson may be drawn from the detection of this fastidious organism only in the 3rd generation of culture and even then only after 1 or 2 preliminary rabbit passages. It was our earlier experience that the cultivation of *Bact. pneumosintes* was more frequently successful from the lung tissues of affected rabbits than directly from the filtered nasopharyngeal washings of influenza patients.

2 other strains of anaerobic, filter-passing organisms morphologically similar to *Bact. pneumosintes* were obtained in Smith-Noguchi medium directly from the filtered nasopharyngeal washings of other

influenza patients. The 1st generations grew so sparsely as to escape detection and the bacteria were first discovered as submicroscopic colonies in subplants on anaerobic blood agar plates. Although these 2 strains have grown well in successive generations on solid media in the anaerobic jar, they have both died out in the Smith-Noguchi tubes and repeated attempts to reestablish growth in fluid media have so far failed. The morphological similarity to *Bact. pneumosintes*, the very minute, discrete colonies on blood agar plates, and the failure to develop in successive transplants on fluid media are characteristic of the Group II organism briefly described by Olitsky and Gates in 1922 (12). These strains have not yet been grown in sufficient quantity for serological examination.

In addition to these 4 morphologically similar organisms, which may all belong to a common group, 2 other anaerobic filter passers were isolated directly from washings of influenza patients. As in the earlier studies, the identification of these 2 other organisms depended on the use of anaerobic blood agar plates on which they grow readily in visible colony form. The primary cultures were obtained in Smith-Noguchi medium; in one instance also in *coli* broth. One strain is apparently a variant of Group I, the other is similar to the organisms described as Group III (12).

Serological Reactions.

At the beginning of this investigation 6 rabbits were set aside for immunization with old strains of *Bact. pneumosintes* (C 17 and C 34) from 1919 and 1922. The organisms were grown in *coli* broth, washed, standardized, and injected subcutaneously in large doses at weekly intervals until 7 or 8 injections had been given. The rabbits then yielded serum with a complete agglutination titer of 1:160 to 1:320 against the old strains. These titers are the highest that have yet been obtained with these organisms and may indicate an increase both in agglutinogenic properties and in response to serum antibodies on prolonged saprophytic cultivation.

The 2 new strains of *Bact. pneumosintes* show a strictly specific agglutination in low dilutions, 1:2 to 1:20, of this anti-*pneumosintes* serum.

At intervals of 15 to 30 days after the acute onset, serum samples were obtained from 8 of the 9 influenza patients from whom washings had been taken, and from 9 other convalescents from clinical influenza. As controls 10 samples were taken from normal persons who said that within a year they had not had any acute respiratory infec-

TABLE I.
Agglutination Tests with Convalescent and Normal Human Sera.

Convalescent sera					Normal sera				
Serum dilu- tions	<i>Bact. pneumosintes</i> strain					<i>Bact. pneumosintes</i> strain			
	17	34	49	50		17	34	49	50
	1:10	1:10	1:2	1:2		1:10	1:10	1:2	1:2
1	+++	++	++	++	1	+++	++	—	
2 ^a	—	—	—		2	+	—	—	—
3 ^b	++	+	++		3	+++	—	++	++
4	++	+++	+	+	4	+++	+	++	+
5 ^c	+++	++	++		5	—	—	—	—
6 ^d	++	++	++	+	6	+++	++	—	
7	++	++			7	—	—		
8 ^d	+++	++	+	+	8	—	—		
9	+++	++	+		9	+++	—	—	—
10	+++	+	++	++	10	—	—		
11	+++	+	—	+	Normal rabbit	—	—	—	—
12	+++	++	—						
13	+++	+	+	++					
14	+++	++	—						
15	++	++	++						
16	++	+	+						
17	++	—							

^a Group III organism recovered.

^b *Bact. pneumosintes* recovered.

^c Group I organism recovered.

^d Group II organism recovered.

tion diagnosed as influenza. These sera were tested for specific agglutinins, by the method previously described (13), against 2 old strains (Nos. 17 and 34), and against the 2 new strains of *Bact. pneumosintes* (Nos. 49 and 50), so far as the very limited amounts of available material permitted.

A summary of these agglutination tests (Table I) shows that the serum of only 1 patient with a clinical diagnosis of influenza failed to

agglutinate 1 or both old strains of *Bact. pneumosintes*, and when tested, most of them (12 of 15) agglutinated 1 or both of the 1926 strains also. Considered together with the agglutination of the new strains by specific anti-*pneumosintes* rabbit serum, this evidence points to an immunological relationship as well as a morphological identity between the 1919 to 1922 and the 1926 strains. Heretofore agglutination of *Bact. pneumosintes* even after a long saprophytic existence in the laboratory has not been found in the serum of supposedly normal persons. In these tests unquestioned agglutination of old and new strains sometimes occurred. Several explanations of this phenomenon are possible, but we shall not attempt to develop any of them at this time, on the basis of the evidence available at present.

SUMMARY.

The presence of *Bacterium pneumosintes* has been demonstrated in nasopharyngeal washings from 2 patients in a sporadic outbreak of clinical influenza in New York City in March, 1926. 2 strains of bacteria morphologically similar to *Bact. pneumosintes*, but differing in certain cultural characters, and 2 other anaerobic filter-passing organisms were also isolated from the 9 patients examined.

The blood serum of 16 among 17 persons convalescent from clinical influenza, and of 6 among 10 supposedly normal persons, agglutinated 1 or more strains of *Bact. pneumosintes*.

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STUDIES ON THE ETIOLOGY OF HEARTWATER.

III. THE MULTIPLICATION OF RICKETTSIA RUMINANTIIUM WITHIN THE ENDOTHELIAL CELLS OF INFECTED ANIMALS AND THEIR DISCHARGE INTO THE CIRCULATION.*

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PLATE 31.

(Received for publication, August 25, 1926.)

The first two of these studies¹ dealt with the discovery of a new species of *Rickettsia* in the tissues of sheep, goats and cattle suffering from heartwater and in the ticks which transmit it. In this third paper, it is proposed to describe in detail the behavior of the *Rickettsiæ* after they have entered the endothelial cells of the blood vessels of infected animals.

The chief obstacle to the acceptance of *Rickettsiæ* as the inciting agents of disease in man is the difficulty of demonstrating their existence clearly and regularly in the lesions. This is almost insurmountable in the case of trench fever, because it is seldom if ever, fatal, so that material for examination cannot be collected post mortem, unless death should accidentally occur at a propitious moment from some other cause, which in practice has not happened. In two other arthropod-transmitted diseases—typhus and Rocky Mountain spotted fever—*Rickettsiæ* are undoubtedly present in the tissues, but only in very small numbers. Their distinctive properties are obscured by extensive vascular degenerations resulting in the formation of many granules which, like them, are colored feebly by basic dyes. Since, moreover, all three species of *Rickettsiæ* are smaller than most

* Sixth contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made and material collected at the Government Laboratories at Onderstepoort and cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler and to the members of his staff for the many courtesies extended. The study herein reported was made after my return to the United States.

¹ Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231, 253.

bacteria, and stain much less intensely, it is not surprising that so little is known of their exact relation to the tissues of man and experimentally infected animals.

It is believed that heartwater affords an unusual opportunity for securing information on this particular aspect of the general problem of *Rickettsiæ* and of their relation to disease.² As already noted degenerative vascular lesions are almost entirely absent in heartwater, and the *Rickettsiæ* themselves, while very sparsely distributed in the tissues as compared with some bacteria in other conditions, are nevertheless much more abundant than the *Rickettsiæ* in any of the above mentioned infections. Another advantage offered by the heartwater *Rickettsiæ* is that they may be colored, without great difficulty, by a variety of stains in addition to that of Giemsa on which chief reliance must be placed for the study of the other *Rickettsiæ*.

Despite the unusual facility with which the heartwater *Rickettsiæ* may be observed and studied, they resemble other *Rickettsiæ* closely in many important particulars. Like them they are very small, Gram-negative organisms (resisting attempts at cultivation on artificial media) which are found principally in the endothelial cells of the blood vessels of diseased animals. All are transmitted by the bites of insects or arachnids, and the conditions to which they give rise are similar in so far as they are acutely febrile, the nervous system is frequently involved and they confer an immunity lasting for several years or for life. The chief point in which heartwater differs from typhus fever and Rocky Mountain spotted fever is in the absence of cutaneous lesions, which is probably correlated with the circumstance already mentioned, that there is but slight vascular involvement.

EXPERIMENTS.

Most of the animals studied were infected by the intrajugular inoculation of blood from cases of heartwater; but some were infected by allowing ticks, carrying the virus, to feed upon them. They were sacrificed at appropriate intervals during the incubation period, and febrile stages, and during convalescence (in the rare cases which did not terminate fatally). A detailed account of the experiments, together with the autopsy reports, has already been given in the first two papers.

Rickettsiæ in the Circulating Blood Stream.

That the virus, and presumably therefore the *Rickettsiæ*, are present in the blood stream during the febrile stages and for about 6 days after the temperature has commenced to decline in animals which have not

² Cowdry, E. V., *Arch. Path. and Lab. Med.*, 1926, ii, 59.

succumbed, was shown by the inoculation of blood into other susceptible animals. But although many attempts were made, it was not possible to discover the *Rickettsiæ* in blood smears. This failure corresponds with the experience of investigators while studying the blood of cases of trench fever and of typhus fever. A few heartwater *Rickettsiæ* were, however, detected within the vascular lumina of tissues examined in sections. The most probable explanation is that they do not occur in the blood stream in large masses but as single individuals, or perhaps in the form of very small clumps (Fig. 7). Obviously even the most careful differentiation of the stain might completely bleach single elements of such small dimensions (only about $0.25\ \mu$ in diameter), whereas the large aggregates within the endothelial cells would probably retain the dye more tenaciously, owing to its reduced rate of diffusion.

Penetration of the Rickettsiæ into the Endothelial Cells.

The actual passage of *Rickettsiæ* through the cell membrane was never seen, but there is reason to believe that several may enter a single cell, most likely in sequence.

The sites of entry and the foci of subsequent multiplication are variable. They may penetrate the cytoplasm at a point where it consists of a thin film almost invisible microscopically, relatively remote from the nucleus. Or, they may gain admittance to the cytoplasm lying between the endothelial nucleus and the lumen of the blood vessel (Fig. 2). In rare cases they may migrate through the cytoplasm to a point on the opposite side of the nucleus, that is to say between it and the periphery, remote from the lumen (Fig. 5).

Multiplication in such a location, proximal to the endothelial nuclei, has not thus far been reported in the case of any of the other pathogenic *Rickettsiæ*. Yet it might be expected to occur, because the *Rickettsiæ* of both typhus and Rocky Mountain spotted fever possess much greater power of penetration than the heartwater *Rickettsiæ*. They even enter the successive vascular tunics and frequently gain an extravascular position, whereas those of heartwater are apparently definitely and invariably confined to the vascular lumen and its lining endothelium.

Before considering the probability of sequence in penetration, reference must be made to an observation which was briefly mentioned in the first paper, namely, that many spherical aggregates of *Rickettsiæ* may develop within a single endothelial cell. Their number may reach as many as eight. In Fig. 1 six are illustrated within the limits of a section only 3 microns in thickness. The colonies invariably remain distinct and never fuse, even when closely applied to each other.

The clumps inhabiting one cell are seldom of the same size. Their dimensions may vary from about $0.5\ \mu$ to $25.0\ \mu$ as observed after fixation and staining, and the number of *Rickettsiæ* from two to several hundreds. Sometimes the *Rickettsiæ* in the smaller ones are slightly larger and color more deeply than those grouped in the larger masses (Figs. 1 and 6). Although such a distinction is by no means of constant occurrence, it does seem probable that the smaller colonies are younger than the larger ones,—that they had less time to multiply—in other words, that there has, in fact, been a sequence in the entry of *Rickettsiæ*.

Just why the *Rickettsiæ* should enter some endothelial cells in marked preference to others is not known. It does not seem to take place *pari passu* with the phagocytosis of broken down erythrocytes, for example. In heartwater there is very little taking up of erythrocytic debris by the endothelial cells of the cerebral cortex, and kidney, which nevertheless harbor *Rickettsiæ* more constantly than the cells of any other tissues. In the rare cases when it is phagocytized in these localities the cells which do so have never been observed to accumulate *Rickettsiæ* as well, but it would be unsafe to assert that such a double acquisition is impossible. It is conceivable that there may be an element of antagonism in the two processes. Thus, the *Rickettsiæ* were never seen in the endothelial cells of the liver, and they are of very inconstant occurrence in those of the spleen, both of which are noted for the avidity with which they phagocytize erythrocytes and materials of foreign origin generally. Neither is the liver the best place to search for the *Rickettsiæ* of typhus and Rocky Mountain spotted fever, although they do occur in it sparingly. Experimental blocking of the endothelium in heartwater with particulate matter might perhaps modify its reaction to the *Rickettsiæ*.

The *Rickettsiæ* of heartwater resemble those of typhus fever in this predilection which they have for the brain, while they differ in other minor points of distribution, such as their failure to accumulate in very large numbers in the skin, or the testicle, and the peculiar attraction which the kidney seems to exercise over them.

Growth of the Rickettsiæ within the Endothelial Cells.

A single isolated *Rickettsia* has never been seen within an endothelial cell; but in Fig. 5 a *Rickettsia* in the act of division, or two *Rickettsiæ* in especially close contact, are illustrated. In very thin sections ($1\ \mu$) it is possible to distinguish similar formations suggestive of simple transverse fission, and it is probable that this process takes place commonly and at a very rapid rate. Sometimes the *Rickettsiæ* are congregated at the periphery of the colony in such a manner as to suggest the interpretation that they divide in a plane perpendicular to its surface and that the growth is peripheral rather than central (Fig. 3).

Some striking examples of growth pressure were found. Fig. 2 illustrates a colony which has developed between the nucleus and the vascular lumen. While growing it has pressed upon the nucleus, forming a marked concavity in it. A rarer instance, which, indeed, has only once been observed so clearly, is still more significant (Fig. 4). It consists of two colonies of approximately the same size (and age) which have developed at opposite poles of the nucleus of an endothelial cell. When the *Rickettsiæ* first commenced to multiply, this nucleus was presumably flattened by arterial blood pressure in a direction parallel to the length of the vessel, like its neighbors. The pressure which the *Rickettsiæ* have exerted has completely altered its position so that it has been forced to stand on its head, so to speak. This can only mean that the growth pressure of the *Rickettsiæ* actually exceeds the arterial blood pressure because it is sufficient to cause a nucleus, which is ordinarily flattened by the blood pressure, to expand directly against it. To the best of my knowledge no alteration like this is produced through the phagocytosis of lifeless materials.

The observation that when two colonies grow toward each other and come in contact the area of separation between them is a plane

surface indicates that their growth pressure is about equal. If it were greater in one than in the other, only one would become flattened (*i.e.*, that in which the pressure was least), or else the one endowed with the largest expanding force would indent the other. It is possible, thus, to ascertain that the growth pressure of small colonies is approximately the same as that of larger ones.

Throughout this period of multiplication the morphology and staining reactions of the *Rickettsiæ* vary only within the limits already alluded to. Figs. 3, 4 and 6 illustrate their appearance by Giemsa's method; Figs. 2 and 5 by hematoxylin and eosin; Fig. 7 by Loeffler's methylene blue; and Fig. 1 after staining with eosin and methylene blue. The variability in color of some of the *Rickettsiæ* is probably caused by fortuitous differences in the rate of extraction of the stains, some of the *Rickettsiæ* being nearer than the others to dye-holding materials or to the surface of the section.

Thus, in their tinctorial properties also, they resemble the *Rickettsiæ* of typhus and Rocky Mountain spotted fever, though they are more easily stained. In all three the differentiation into red- and blue-staining materials, which may be observed in smears of the arthropod vectors, is not distinguishable in sections of infected mammalian tissues.

That the *Rickettsiæ* of heartwater are organisms possessed of definite internal organization, is shown by the fact that they retain their spherical shape and do not tend to flatten out when they come in touch with the cell wall and other cellular components. Yet with a very high magnification (of 4000 diameters or more) their outlines become more hazy than do those of the mitochondria, for instance. This would lead one to suppose that, if they possess an organized limiting membrane, it is of somewhat rudimentary character as compared with that of typical bacteria.

Only in an animal's tissues which have not been promptly excised after death, are any signs of the degeneration of *Rickettsiæ* to be noted. When, under such conditions, degeneration sets in the *Rickettsiæ* begin to exhibit considerable variability in size and to lose their peculiar tinctorial properties. The forces which have previously kept them spacially arranged in respect to their fellows cease to operate and they tend to agglutinate in rather amorphous masses. Though

unquestionably dead, they may still be recognized for 6 hours or more after the death of the animal containing them, chiefly by their topographic relationship to the blood vessels.

Reaction of the Endothelial Cells to the Presence of the Rickettsiæ.

Unless the living endothelial cells contain *Rickettsiæ*, they show no signs of injury. In this respect the endothelial reaction in heartwater differs sharply from that which may be observed in typhus and Rocky Mountain spotted fever. In these two diseases the endothelial cells in which the *Rickettsiæ* are absent become altered almost as regularly and to about the same degree as those which harbor many of them.

When the *Rickettsiæ* of heartwater do enter the endothelial cells and multiply within them, they do not cause very serious injury. The principal alteration which the cells undergo is great mechanical distention to accommodate the *Rickettsiæ* in large numbers. Their nuclei seldom, if ever, show definite signs of injury. Karyokinetic figures are of very rare occurrence and there is no evidence of amitosis. It is possible that the mitochondria and Golgi apparatus in the cytoplasm are altered; but they cannot be greatly damaged, because the cells continue to live. No signs whatever of basophilic degeneration, like that which occurs in typhus and Rocky Mountain spotted fever, were seen. Indeed, the cytoplasm, although swollen and greatly increased in extent, reacts in the usual way to all ordinary stains except for certain foci, in which the *Rickettsiæ* are multiplying, which become chromophobic.

These vacuole-like spaces containing *Rickettsiæ* are filled with a clear watery fluid, probably saline in nature, as the action of a wide range of fixatives fails to reveal any coagulable substance in it. The margins of these spaces are sharply defined and do not grade into the surrounding cytoplasm. Morphologically the spaces do not differ from areas of intracellular digestion containing broken down red blood cells, but biochemically they are probably dissimilar, for the reason that there can be but little in common between the process of intracellular digestion and the conditions which facilitate the multiplication of *Rickettsia*-like organisms.

The *Rickettsiæ* of heartwater are usually observed clumped in the central parts of these spaces. They do not become applied to

the interface between the fluid contents of the spaces and the cytoplasmic environment. It is interesting to note that neighboring spaces communicate with one another, as illustrated in Figs. 1 and 6; also, that a single space may house more than one colony of *Rickettsiæ* (Fig. 1).

The reason why these colonies of *Rickettsiæ*, suspended in a fluid medium within a single vacuole-like container, or communicating system of containers, of microscopic dimensions, should retain their individuality instead of coalescing to form a larger mass, is not easily discovered. One would expect them to be thoroughly mixed by the continual changes in size of the pulsating vascular walls, but they are equally discrete in veins, which do not pulsate, as in arteries.

In sectioned mammalian material, it has not been possible thus far to bring to light any indications of the existence of a matrix, or binding material, which might restrict or retard the movement of the individual organisms forming a colony. But, in the second paper, on the *Rickettsiæ* of heartwater as they appear in infected ticks,³ some clumps of *Rickettsiæ* were found to be embedded in such a ground substance which differed slightly in staining properties from the cytoplasm of the host cells. This was best seen after formalin fixation. When, moreover, portions of the tick's alimentary tract were teased out in an approximately isotonic medium the individual *Rickettsiæ* in a clump were found to cohere together despite considerable mechanical traction. Two possibilities therefore present themselves; either a matrix of this kind is absent in the tissues of infected mammals, or else it is present in such a form that it eludes our methods of demonstration. It may be an inconstant accompaniment of intracellular *Rickettsial* growth, and exist in very small amounts in the case of *Rickettsia prowazeki* in which the "globular massing" is so much less distinct.

Discharge of Rickettsiæ from the Endothelial Cells into the Blood Stream.

After the incubation period has passed and the febrile reaction is at its height, the endothelial cells often rupture and discharge their contents into the circulation (Figs. 4 and 6). Sometimes the broken

³ Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 253.

edges of the cell membrane may be distinguished so that it is certainly not a case of the entry of *Rickettsiæ*. That we are not dealing with a dragging out of the *Rickettsiæ* by the microtome knife (as occasionally happens in the case of resistant bodies, like nucleoli) is indicated by the absence of scratches and by the observation that in one and the same section the discharge may take place in several directions. As far as our microscopic evidence goes, these liberated *Rickettsiæ* have every appearance of being just as viable as those which originally entered the cell. No parallel instance of the discharge of *Rickettsiæ* into the blood stream has been reported in Rocky Mountain spotted fever or in typhus fever. A special search which I have made myself of tissues very kindly given to me by Drs. Wolbach, Rajchmann and Nicholson has failed to reveal any indications of a similar process.

Even with localized cellular injury of this extent and degree, the nucleus remains apparently normal and the cell continues to live. This normality of the endothelium constitutes another point in favor of the conclusion that the *Rickettsiæ* live and actively multiply within the vascular endothelial cells. By contrast, the extensive endothelial injury in typhus and Rocky Mountain spotted fever seems to be correlated, at least in part, with the death of many *Rickettsiæ* within the cells and the resultant liberation of substances which may be poisonous. In other words, the endothelial inclusion of *Rickettsiæ* is not so much a mechanism of defense in heartwater as it appears to be in these other diseases.

DISCUSSION.

The *Rickettsiæ* of heartwater exhibit certain features typical of other pathogenic *Rickettsiæ* with almost exaggerated clearness.

The most characteristic appearance of *Rickettsiæ* in human lesions is, according to Wolbach, Todd and Palfrey,⁴ their tendency to globular massing. In heartwater this property of forming spherical clumps within the endothelial cells is even more conspicuous. These spherical masses, or colonies, grow within vacuole-like cytoplasmic spaces filled with a clear fluid. Further data are needed concerning these spaces, because clues might thereby be secured for the preparation

⁴ Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

of artificial media suitable for the cultivation *in vitro* of the heartwater *Rickettsiæ* and conceivably for those of typhus and Rocky Mountain spotted fever also. Many difficulties would be encountered in making such an analysis, but indicators might be of service, especially the *in vivo* synthesis of Prussian blue. The oxydase reaction should be applied. The fact that multiplication appears to attain a maximum during the height of the febrile period, when the temperature often reaches 107° or 108°F. may be significant taken in conjunction with the fact that multiplication is also rapid in the body of the tick at a temperature about 30° lower. Additional studies on the matrix which seems to bind together the individual *Rickettsiæ*, which compose a colony, might also bring to light information regarding a medium which would justify further work along the lines of artificial cultivation.

Another feature which all pathogenic *Rickettsiæ* possess in common is the habit of becoming parasitic in the vascular endothelia. This is the usual location in human and mammalian tissues of the *Rickettsiæ* of Rocky Mountain spotted fever, in which condition, however, they may spread to the media and adventitia and occasionally to parenchymatous elements such as liver cells.⁵ The *Rickettsiæ* of typhus fever are somewhat more restricted, being found only in the mononuclear cells of the perivascular nodules in addition to the endothelium; while the *Rickettsiæ* of heartwater are the most specific of all, since their location is confined, as far as can be ascertained, without exception, to the endothelium.

This restriction of a pathogenic microorganism to a single type of cell has recently been claimed for the tubercle bacillus by Sabin and her coworkers.⁶ She believes that the tubercle bacillus attacks a specific kind of cell—the monocyte—penetrates into its interior and multiplies within it. The evidence for intracellular multiplication is conclusive. Maximow⁷ has observed the process in the living cells of tissue cultures; but he has found that other cells, as well as the monocyte, are invaded, and some believe that the monocytes do not differ fundamentally from

⁵ Nicholson, F. M., *J. Exp. Med.*, 1923, xxxvii, 221.

⁶ Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.

⁷ Maximow, A. A., *J. Infect. Dis.*, 1924, xxxiv, 549.

the macrophages,⁸ or clasmatoocytes, as they are often called. The case of heartwater is more concise, for there can be no dilemma in the identification of endothelium. While the *Rickettsiæ* of heartwater may apparently live and multiply within the endothelial cells, there is no evidence to indicate that their action stimulates the endothelial cells to divide and increase in number in a fashion comparable with the influence exercised by ingested foreign materials, such as the tubercle bacilli, upon the monocyte.

It is interesting to note that, although in heartwater the endothelial cells remain living and normal and are so heavily charged with *Rickettsiæ* that they may be considered to be marked by their presence, the free large mononuclear cells of the circulating blood (monocytes, endothelial leucocytes, etc.) which Mallory and his associates believe to be developed directly from the endothelium, remain wholly devoid of *Rickettsiæ*, or at least contain *Rickettsiæ* so infrequently that they have thus far escaped observation.

SUMMARY.

The *Rickettsiæ* of heartwater are more definitely restricted to the vascular endothelial cells of infected animals than are those of typhus or Rocky Mountain spotted fever. They likewise form more pronounced spherical colonies within the cells. Their presence does not injure the endothelial cells to a degree at all comparable with that caused by the other pathogenic *Rickettsiæ*. The rupture of endothelial cells and discharge of *Rickettsiæ*, which are apparently viable, into the circulation constitute phenomena not thus far reported in the case of typhus or Rocky Mountain spotted fever.

EXPLANATION OF PLATE 31.

All the figures were drawn at the level of the table with a 1.30 aperture apochromatic objective of 1.5 mm., compensating ocular 18 and camera lucida. They have been reproduced without reduction so that, as they appear, they represent a magnification of 4033 diameters. They have all been made from tissues of Goat 4510.⁹

⁸ Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

⁹ For details of temperature reaction and postmortem examination, see Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231.

FIG. 1. Cerebral cortex fixed in Regaud's fluid and stained with eosin and methylene blue. Several colonies of *Rickettsiæ* are seen in the cytoplasm to the left of the nucleus. They are of variable size. In the smallest, a single organism is represented which is slightly larger than the others, is colored rather more intensely and seems to be in the act of division.

FIG. 2. Cerebral cortex fixed in Regaud's fluid and stained with hematoxylin and eosin. The *Rickettsiæ* are feebly colored. They are contained in an unusually large vacuole-like space (distal to the nucleus, *i.e.*, between it and the lumen) the upper margin of which can be clearly distinguished. Through growth pressure they have indented the nucleus.

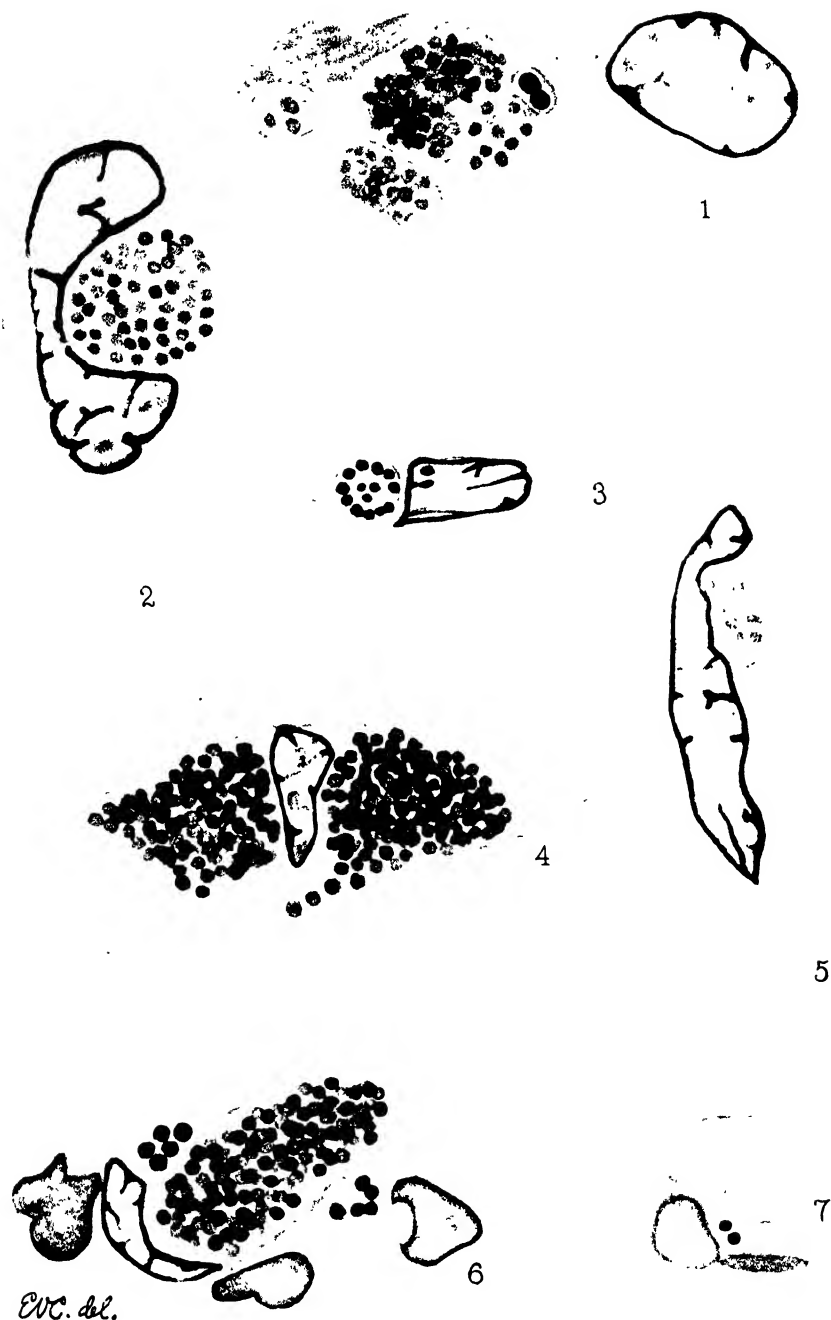
FIG. 3. Cerebral cortex fixed in Regaud's fluid and stained by Giemsa's method. A colony of *Rickettsiæ* is represented at one pole of the nucleus. Organisms which seem to be dividing are oriented with plane of division at right angles to the margin of the vacuole.

FIG. 4. Pancreas fixed in Regaud's fluid and stained by Giemsa's method. There are two colonies of *Rickettsiæ*, one on either side of the nucleus. Their growth has exerted sufficient pressure to cause the nucleus to alter completely its position by projecting directly toward the lumen in a direction parallel to pressure exerted by the blood stream. A few of the *Rickettsiæ* are being discharged into the lumen.

FIG. 5. Cerebral cortex fixed in Regaud's fluid and stained by hematoxylin and eosin. Here the *Rickettsiæ* are multiplying in a very unusual position, proximal to the nucleus and remote from the lumen.

FIG. 6. Cerebral cortex fixed in Regaud's fluid and stained by Giemsa's method. Two colonies of *Rickettsiæ* are illustrated. Those comprising the smaller one are stained more intensely than the others and are noticeably larger. Some *Rickettsiæ* are escaping from the larger colony into the blood stream.

FIG. 7. Cerebral cortex fixed in Regaud's fluid and stained with Loeffler's methylene blue. Two *Rickettsiæ* embedded in some chromophobic material are seen in the lumen between the erythrocytes.



THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VI. FACTORS DETERMINING THE REACTION OF SKIN GRAFTS; A STUDY BY THE INDICATOR METHOD OF CONDITIONS WITHIN AN ISCHEMIC TISSUE.

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PLATE 32.

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In previous papers from this laboratory a technic has been described for the examination of tissues vitally stained with litmus or with indicators of the phthalein series (1), and some observations on the apparent reaction within various organs have been recorded (2). Recent control tests on the influence of tissue materials upon the colors manifested by phthaleins staining them have shown that these colors are not conditioned to any important degree by "salt and protein errors," but that they are really indicative of the prevailing pH (3). By means of vital staining with selected indicators one should be able to apprehend, and may even come to understand, certain physiological states inaccessible to study by approved quantitative procedures. One such state, that of "outlying acidosis," has already been briefly reported upon (4). The present paper is concerned with the conditions in a tissue, the skin, which survives when its blood supply has been cut off. The problem has both practical and theoretical ramifications. In corollary to it the changes which result from superimposed injuries to the tissue elements will be considered.

The Vital Staining of Skin Grafts with Phenol Red.

Male white mice of 25 to 30 gm. were shaved over the sides and back, from fore legs to hind, and under ether anesthesia pieces of the skin were excised, midway between axilla and groin, and at once sewn back in place again. These pieces were roughly circular and from 1.0 to 1.6 cm. in diameter. They had the entire

thickness of the integument and contained, in addition to epidermis and corium, a varying quantity of fat and the thin layer of striped muscle which in the mouse and rat extends superficially over almost the whole body. It was frequently impossible to remove such large pieces without cutting cutaneous vessels that supplied neighboring regions, and in consequence of this occurrence the grafts often failed to become vascularized promptly and perished after some days. In females the mammary gland proved a serious complicating factor, and after a few trials males alone were used. The best results were had when the graft was not separated all at once from its surroundings but left attached by an isthmus that was severed only after the major portion had been sewn in place. By this procedure the swabbing of the raw surfaces with saline solution to prevent drying was reduced to a minimum; but great care had to be taken in the dissection else some of the loose web of tissue connecting the skin with the underlying parts escaped being cut, with result that total ischemia of the graft did not develop. For the sewing a fine curved needle carrying a single strand of the three which are wound into No. 2 surgeon's silk was employed; and an over and over' stitch. Perfect approximation of the wound edges proved essential, for when a part of the wound bed was left exposed necrosis usually spread from it (5). Aseptic conditions were maintained during the operation; and no dressing was put on afterwards. The mouse had to be prevented from removing stitches and graft however. This was accomplished by passing its head through a hole in the center of a flat disc of pasteboard. The projecting collar thus formed, which stood out like the ruff of a clown, did not interfere with the animal's movements but kept it from gnawing at the graft. The stitches were removed on the day following the operation, and thereafter for nearly a week the attachment of the implanted skin was but frail.

Each day the mouse was injected into the peritoneal cavity with 0.5 cc. of a watery 4 per cent solution of phenol red, made as follows: 2 gm. of the phthalein (Hynson, Westcott and Dunning) is ground to a paste with a little water, 9.7 cc. of $N/1$ NaOH added, and then water to 50 cc. Such a solution is at pH 7.4 (as determined with the potentiometer) and approximately isotonic with 0.9 NaCl. The quantity of alkali employed somewhat exceeds that required, on theory, to bring the indicator to the hydrogen ion concentration mentioned, presumably because of acid impurities in the material. In order to effect the injection into the mouse without a struggle that might have entailed separation of the graft it was briefly anesthetized by dropping it into a jar containing cotton soaked in ether.

During most of the 1st week after implantation the graft appeared pallid,—save when vitally stained,—and bloodless. As is well known vessels begin to penetrate into transplanted skin during the second 24 hours, but there is certainly no effective circulation of blood for a much more considerable period in grafts of the size studied in the

present work. This is evident not merely from the aspect of the graft under ordinary conditions but from the slowness with which it colors up after an intraperitoneal injection of phenol red. On the 3rd day after operation it stains no more rapidly than on the 1st. Thereafter, though, it lags in this respect less markedly as compared with the skin round about, and by the end of a week, in successful instances, it colors as fast as the latter.

The rate of coloration provides enlightening data on the fluid interchange taking place within engrafted tissue. Phenol red is very highly diffusible, coloring mice deeply within a few minutes after an intraperitoneal injection; and the brilliant, ruddy color of the stained animals is due for the most part to an extravascular penetration of the dye. This has been shown by perfusing the stained animal until free of blood, with warm Locke's solution introduced into the beating heart, after the inferior vena cava has been snipped across (6). But the fact emerges even more strikingly from the observations on the skin grafts of the present work. These color deeply, evenly, and surprisingly fast with the phthalein at a time when it is certain no blood can reach them, that is to say within a few minutes after they have been separated and sewn in place again. The hue they manifest is referable to staining of the subepidermal tissue fluids and tissues, especially the corium. Muscle stains but slightly with the dose of indicator I have employed; fat scarcely at all; while the epidermis is so thin and so lightly stained that it may be dismissed from account.

In recording the hues of animal and graft Ridgway's "Color standards and nomenclature" (7) has proved of great service. Wherever the hues provided by this book are mentioned in describing the findings italics will be employed. For the matching, one of the standards at a time was exposed through a hole cut in a sheet of white paper, and compared with the skin color. A normal mouse of 28 gm. given 0.5 cc. of phenol red ordinarily becomes deeply stained within 15 minutes, and reaches a maximum color, one varying between *jasper red* and *eugenia red*, about 30 minutes after the injection. The healthy avascular graft usually remains entirely unstained for from 10 to 15 minutes (Fig. 1) after the injection,—a staring, pallid patch in the midst of the red body surface,—and then it begins to turn yellow here and there. The staining reaches a maximum intensity, *apricot*

orange ordinarily, after a little less than an hour in all, and then appears even (Fig. 2). The hue of many mice will already have begun to fade, owing to elimination of the phthalein into bile and urine; but long after the general decoloration has taken place, a process usually completed by the end of $2\frac{1}{2}$ hours, the graft remains brilliantly tinted (Fig. 3). It is still yellow more than 5 hours after the injection; for the dye leaves the avascular tissue far less rapidly than it enters it.

These are the happenings whenever the animal is stained during the first 3 or 4 days after the implantation,—and it can be stained again and again without evident injury to either it or the graft. Later, as the new vessels become effective and the penetration of the phenol red into and out of the implanted tissue does not lag so noticeably, the color of the graft comes day by day to have less of yellow and more of red in it. By the end of a week the “take” is usually perfect, and the phthalein coloration differs practically not at all from that of the host.

Abnormal Reaction of the Grafts.

The color of the stained graft seems to indicate that it is acid as compared with the normal skin round about; and there is every reason to believe that this is the actual case. Phenol red is a stable indicator, not liable to error through its association with tissue components (8) with the important exception of the proteins of plasma. Kendall (9) states that some destruction of it by reduction occurs within the organism; but the amount changed in this way is negligible when large quantities are given, as was the case in the work here reported. Dr. D. R. Drury, of the laboratory staff, has recovered from the urine of a rabbit more than 96 per cent of the phenol red required to stain it vitally, and a part of the missing 4 per cent was present in the feces, which were not extracted. For nearly 40 years (10) the fact has been generally recognized that acidity develops in tissues when their blood supply is interfered with. If a string is tied tightly around the shaven leg of a rat stained with phenol red the color of the leg turns from red to orange within a few minutes, only to become red again shortly after the cord is loosened and circulation resumed. If one evert a flap of living subcutaneous tissue vitally stained with the dye it turns from

red to purple in proportion as carbon dioxide escapes from the raw surface; and the purple can be converted to orange-yellow by brief exposure of the tissue to an atmosphere of CO_2 (11). These are simple instances illustrating the readiness with which the phthalein reacts under *in vivo* conditions and showing that it reacts characteristically.

One can appraise the color of the stained body surface of the living animal by oiling it, placing over it an Autenrieth wedge filled with water, and comparing the color as thus viewed with that obtained by superimposing a similar wedge, filled with an appropriate buffer solution colored with phthalein, over the oiled and shaved skin of a normal animal. By varying the buffer solution and moving the colored wedge until precisely the right depth of fluid is obtained, one can closely approximate the hue of the stained tissue. *Apricot orange* corresponds with a pH of about 6.8, *jasper red* with pH 7.4, and *eugenia red* with pH 7.6. The actual figures can be disregarded. It is their relation to each other which tells the story. Evidently vigorous skin grafts of the mouse are, relatively speaking, about pH 0.6 more acid than the normal skin, and they are able to survive this state of affairs for several days. The acidity is referable, at least in the beginning, to the elements proper to the tissue, not to the many cells that wander in (12), as is sufficiently shown by its development within an hour after implantation of the graft.

Vigorous Grafts Are Acid, Weak Ones Alkaline.

At an early period in the work, when the operative technic was uncertain, grafts coloring red with the phthalein were not infrequently encountered. It was natural to suppose that these, as manifesting a tissue reaction close to the normal, would be the ones to survive; and not until some thirty orange or red examples had been followed did the fact emerge that it was precisely those grafts which showed for days the orange color of an abnormal acidity that lived and healed into place. Whenever the implanted bit of skin, or a portion of it, repeatedly stained red during the early period after operation when it should have been reestablishing itself, that graft or portion was noted soon after to perish. Once this had been realized reasons for the color difference were not far to seek. Owing to poor fluid interchange the acid products of the cell metabolism of healthy grafts would tend

to heap up, just as they accumulate in subcutaneous areas temporarily deprived of circulation through the vascular contraction following on a local injection of epinephrine (13). The cells of injured tissue on the other hand not only work less actively, or dying, cease to work at all, but to a greater or less degree they lose that semipermeability which characterizes them during life. As result they are penetrated by the alkaline lymph which acts to sweep away such acid products as may arise through autolysis, and, coming gradually into equilibrium with this fluid, they tend to approach it in reaction. One may doubt whether the interstitial fabric which forms so large a part of the corium, though staining deeply with the phthalein (14), possesses life of its own in any proper sense. One must think of its reaction as determined preponderantly by the activities of the cells dispersed through it.

Grafts Injured Experimentally Are Alkaline.

To test this explanation of the findings pieces of skin of the usual size were damaged prior to implantation. The intention was to inflict the minimum insult that would ensure an eventual failure of the graft. Heat was employed in some cases, but repeated freezing and thawing proved better for the purpose.

In order to heat the pieces of skin they were folded upon themselves with the raw surface inwards, and placed far down in test-tubes already in a water bath at 50–51°C. The grafts adhered to the sides of the tubes which had moist gauze at the bottom to prevent drying. They were heated for from 7 to 10 minutes.

When a graft was to be frozen and thawed it was spread upon a sterile mica slide with its raw surface against the latter. To prevent drying the edges were sometimes folded under; but more usually a drop of salt solution, or Ringer's solution, was run around the edge of the tissue. The slide was then placed on the freezing disc of a microtome, the tissue frozen solid by the escape of CO₂, and the preparation removed and thawed at once with the warmth of the hand or that of a metal plate at about 38°C. The processes of freezing and thawing were carried out three or four times as rapidly as possible, and the graft was replaced in position. Meantime the skin defect on the body of the animal was covered with a sponge moist with salt solution or Ringer's solution.

It proved easier to return the grafts to their original position when they were asymmetric or had been cut with a slight projection at one point in the periphery that fitted into a notch in the skin.

Heating to a temperature of 50°C for 10 minutes should have killed the tissue,

under the conditions. Such heat regularly results in an eschar after 2 days, when applied to the shaved body of rats by way of a glass disc through which hot water circulates. But it was only after the lapse of about a week that the heated grafts became evidently necrotic. Until then they maintained the aspect of life, being pliable though definitely swollen and somewhat more opaque than control grafts on the other side of the same animal. They continued to be pallid, however, long after healthy grafts had begun to show the flush of a renewed circulation; and eventually they dried, remaining adherent over the advancing edge of the skin as it encroached on the defect beneath them.

If the appearance of the heated grafts was for a long time much like that of surviving tissue this was never true of their reaction. From the moment that they were sewn back in place they were always frankly alkaline to phenol red, as alkaline to appearance as the surrounding normal tissues and occasionally somewhat more so, being then of a more purple hue (Fig. 2).

Repeated rapid freezing and thawing, unlike heating, failed to render a graft more opaque than ordinary, and when first sewn in place it had precisely the appearance of the control graft on the other side of the same animal. Yet freezing and thawing is known to kill mammalian cells of many sorts; and I have repeatedly utilized it to destroy the cells of a transmissible chicken sarcoma without injury to the filterable agent responsible for the growth. It did cause eventual failure of the skin grafts. These retained their appearance of life for a week or 10 days, though, and often developed what appeared to be a surface union with the surrounding tissues. They never vascularized, however, but remaining pallid, and, becoming gradually thinner and parchment-like, they dried up. Unlike heated grafts they were never in the least edematous. From first to last the frozen and thawed skin stained red with phthalein, often a more purple-red than the animal.

It was, as has been stated, a part of the plan of the experiments to injure the implanted skin only to the extent necessary to insure a failure to "take." For, obviously, greater tissue changes would have lessened the chance that the graft would remain placed and in condition to take the stain, and have complicated the interpretation of the results. That the heating was close to the critical amount appears from the fact that a graft submitted to only 48°C. for 7 minutes

stained *apricot orange*, as does a healthy graft, immediately after it has been replaced in position, *coral red* 24 hours later, and it only attained to the *jasper red* of the surrounding body surface on the 2nd day after operation. The long persistence of the frozen and thawed grafts and their temporary union with the surroundings also bespoke the minimum damage compatible with the purpose in hand.

The experiments support the view that the orange staining of the grafts destined to "take" is referable to acid products arising out of the life processes taking place within them, and that the ruddy hue of grafts doomed to fail results from an impairment of such processes.

The Altered Permeability of Injured Grafts.

In not a few instances the avascular implants injured by freezing colored up more rapidly with phthalein than did control implants on the opposite side of the animal (Fig. 1). The latter, though, were somewhat edematous which might well have interfered with the fluid exchange; while furthermore the orange color of the phenol red penetrating into them was not so readily to be perceived as the purple rose in skin that had been frozen and thawed. For these reasons the difference in the rate at which the grafts stained could not be certainly ascribed to a loss of semipermeability of the injured tissue; and it was necessary to resort to special experiments, now to be described, to determine whether such a loss had occurred. The injured grafts retained phenol red for quite as long a period after decolorization of the host (Fig. 3) as did their healthy fellows—a fact which would indicate that the acidity developing in the latter did not lead to any unusual fixation of the dye upon them, like that encountered by Kendall in another relation (15).

For the tests of relative cell permeability in healthy and injured implants erythrolitmin has been employed. The color of animals vitally stained with this indicator is due in the main to an intracellular storage of it; and through local changes in the hue one is enabled to perceive at once when the barrier of semipermeability normally maintained by the cells is broken down. The highly diffusible phenol red is not segregated within cytoplasm, under ordinary conditions at least, and hence it could not be utilized for the work.

Rats and mice repeatedly injected with litmus, or with erythrolitmin, the effective constituent of Kahlbaum's cube litmus as at present available, have much of the indicator segregated within the cells of the subcutaneous tissue, though there is also some diffuse distribution of it throughout the intercellular material. The diffusely distributed litmus is always in the alkaline or blue form, whereas the segregated is red (16). As result of a combination of the two sorts of staining the living animal appears violet-pink for some weeks after injection. Everywhere throughout the corium one finds microscopically that the cells are crowded with pink or red globules, while the intercellular fabric has an even, light blue tint. Injury to the individual cells results in an immediate color change of the globules from red to blue, and the litmus diffuses from them secondarily, staining the cytoplasm and nucleus blue (17). A similar secondary staining under such circumstances with stored vital dyes having no indicator properties has long been familiar to cytologists; and it is plainly referable to a loss of the normal semipermeability. The change from red to blue of the litmus-containing intracellular granules which occurs prior to diffusion of the dye from them is traceable to a like event, the acidity that had prevailed within the segregated material being lost as the alkaline tissue fluids penetrate to it through the cytoplasm. Any gross trauma to the skin of the stained and living animal results at once in a change in the color of the region affected from violet-pink to a sharp blue.

For the purpose of the present tests mice were three times injected into the peritoneal cavity at intervals of a day or more with 0.35 cc. of a 2 per cent solution of erythrolitmin in 0.9 per cent NaCl.¹ Between 1 and 2 weeks later skin grafting was carried out, according to the accustomed technic; but the graft was frozen and thawed three or four times before it was sewn into place. In several animals a control graft of the same size was removed from the other side and replaced at once.

When taken for freezing the graft had the same color as the rest of the integument—a hue approximating the *light vinaceous lilac* of Ridgway (Fig. 5). But immediately that it had been frozen and thawed it changed to a brilliant blue, *Blanc's blue* or *Yale blue* (Fig. 5); and this hue it retained as long as it continued to fill the skin defect. This it did for the period usual with such frozen and thawed material, remaining flexible, and translucent for a week or more, during which period a smooth union with the neighboring skin often appeared to take place.

Despite the most careful handling the healthy graft placed on the other side of the animal also changed somewhat from the hue it had prior to separation. Wherever it was held with forceps or thrust through in the course of the suturing it became blue, as did also the normal skin so treated; and in addition, after it was sewn back, it was noted to be somewhat bluer throughout than its surroundings were (Fig. 5). Next day this unusual color was still as pronounced as at first, and

¹ The erythrolitmin was prepared by a modification of the older methods, which will be described by Dr. P. D. McMaster in a forthcoming paper.

only toward the end of the 1st week, as the graft became established, did the hue gradually revert to the "normal."

The expectation had been that the blue erythrolitmin liberated in the frozen and thawed graft by death of the cells would be carried off little by little in consequence of the local fluid interchange, and that in consequence the color would become much lighter. But, as just mentioned, even the relatively slight amount of blue pigment liberated in the healthy graft persisted as such in it for several days. Erythrolitmin has an affinity for intercellular substances, which remain blue for months after the coloration has disappeared elsewhere (18); and it is highly colloidal, passing to and from the tissues with difficulty. In the light of these facts there is no need to invoke chemical alterations in the fabric of the several times frozen graft to explain its enduring blue color.

The observations demonstrate that the cells of grafts frozen and thawed repeatedly lose their semipermeability. That the change takes place everywhere and all at once may be doubted however: for not only do the grafts retain the aspect of life for many days but their color by transmitted light, prior to implantation after the injury, is ruddy here and there, showing that some of the erythrolitmin still persists in the red form. Only by reflected light is the hue a deep, clear blue.

Permeability of Skin Grafts for Carbon Dioxide.

The increased permeability of the damaged, avascular skin graft has been shown in another way, namely by submitting the body of a mouse carrying it and a healthy graft to an atmosphere of carbon dioxide. The gas penetrates the injured skin with immense rapidity, rendering it acid.

It was Lavoisier himself who first showed that carbon dioxide passes out of the intact skin of mammals; and his observations have been often repeated, with variations. When the skin is hyperemic and moist a not inconsiderable gaseous interchange may take place through it, as much as 4 per cent of the total CO_2 being eliminated in this way (19). To the present no experiments seem to have been made on the penetration of CO_2 from without. Skin grafts *in situ* are ideal for a study of the phenomenon since their avascular condition creates an opportunity for the gas to accumulate within them, as it cannot to any considerable degree within the normal integument.

For the purpose of the tests there has been utilized the funnel gas chamber devised for a study of the changes in pH occurring in raw tissue surfaces (20). The CO_2 was let in at *D* in the apparatus as figured in the paper referred to, and

it escaped, in some part, through the other tube *E*, which had now been provided with a connection leading off to the floor. The mouse lay on top of two layers of rubber dam which in turn were spread upon an electrically heated pad. The animal had been stained with phenol red as usual and rendered quiet by the intraperitoneal injection of 0.15–0.2 cc. of 20 per cent urethane shortly before the observations were begun. These latter were only undertaken 2 days or more after the body surface of the animal had been shaved and the grafts placed, in order to rule out the possibility of any entrance of the CO_2 through surface abrasions. The hair had been removed from about the neck of the animal with a sodium sulfide solution; and a round hole in the rubber dam closing the gap at the margin of the funnel fitted the neck snugly. The edges of the dam were everywhere attached to the funnel by adhesive except below, where the rubber extended along the pad in a broad apron ending 6 to 10 cm. away from the head of the mouse. In order to rule out any possibility that the animal might inhale CO_2 , a second piece of rubber dam was placed about its neck, over the first, to block off the gas chamber more completely; and the head was thrust into a small funnel through which a continuous gentle stream of air was blown against the nostrils. Tests with smoke after the experiments showed that some CO_2 escaped from beneath the funnel here and there, as well as through the proper outlet for it, but that none whatever could have been inhaled. A thermometer was introduced into the gas chamber through separate piercings in the layers of rubber dam, with the bulb lying between the groin of the mouse and the heating pad. The temperatures ranged between 36° and 38°C .

When an atmosphere consisting entirely of CO_2 was to be used the gas was led in from an ordinary Kipp generator after passage through a wash-bottle. The water in this latter never contained more than a trace of HCl , and there would seem to be no possibility that the effects on the skin surface of the mouse can have been due to another cause than carbon dioxide. The rapidity with which they disappeared when the surface was once again exposed to air also bespeaks the action of the gas. In some special experiments a mixture from a compression cylinder, containing 10.35 per cent CO_2 and approximately 20 per cent O_2 , and 70 per cent N_2 was employed. Dr. C. A. L. Binger kindly determined the percentages.

When comparisons were to be carried out with the Ridgway color standards the stopper was briefly removed from the top of the gas chamber so that the inspection could be made without the intervention of a glass wall. Access to the body of the mouse could also be had in this way without changing the gas. To replace the latter with air a tube was thrust in and the chamber emptied almost instantaneously with the aid of the laboratory vacuum.

On running pure carbon dioxide into the chamber a change could be noted practically at once in the color of the frozen and thawed, avascular graft. It began to turn from *eugenia red* through *apricot orange* to a brilliant untempered *orange*, reaching this hue within 8 or 10 minutes (Fig. 4). Further exposure to

CO₂ did not alter the color for the good reason that the acid end of the range of phenol red had been attained. The control graft on the opposite side of the animal was *apricot orange* or *apricot buff* to begin with; and during the brief period in which the frozen and thawed tissue was running the gamut from purple red to orange it altered slightly, to *zinc orange*; but it obviously lagged in changing color as compared with the injured tissue and was still definitely less orange than the latter after 15 minutes, the maximum period of the observations (Fig. 4). On the other hand when once again exposed to the air it kept its unusually intense orange color long after the frozen and thawed skin had once again become *eugenia red*. This happened within about 15 minutes.

During the time that the grafts were undergoing these changes a slight but definite alteration was to be noted in the color of the body surface generally. It turned from red toward yellow, that is to say from *eugenia red* to *jasper red* in the case of animals approximating the first mentioned hue, and from *jasper red* to *carrot red* in some other individuals. There is, by the way, not a little variation in the surface hue of normal animals stained with phenol red, as the mention of these differing initial colors will attest. Variations in the normal pH of the blood, similar in magnitude to those here indicated by the phthalein have, of course, long been recognized to exist.

In order to determine whether the general change in color of the animals submitted to CO₂ was referable to absorption of this gas by way of the grafts or to a passage of it through the undisturbed skin everywhere, normal mice were shaved from fore legs to hind and 2 days later were exposed to pure CO₂. There ensued changes in the general hue identical with those just described. At the end of three-quarters of an hour they were no more marked than after 15 minutes. They were indicative of an apparent fall in pH from about 7.6 or 7.5 to 7.4 and 7.3 respectively. The rapidity with which the color reverted to the "normal" when the mouse was once again exposed to air was startling. Within 3 minutes the change had been completed. The surface acidosis described evidently resulted from a continuous passage of CO₂ through the skin, one not entirely compensated for locally, in the surface regions at least, by circulatory and respiratory readjustments. In a number of animals the rate and amplitude of the breathing were followed throughout the experiment. Changes accompanied the exposure to CO₂, and doubtless analyses of the expired air would have shown that no inconsiderable quantity of the gas, in addition to that resulting from body processes, was being given off through the lungs.

Water never condensed within the funnel chamber out of the CO₂; and the skin of the mice was as dry to the feel as ordinary. The findings certainly cannot be laid to the presence of an abnormal amount of moisture on the skin surface. Moistening the grafts led, as was to have been expected, to a more rapid penetration of CO₂ into them. To demonstrate this drops of distilled water were placed here and there on the skin and on the grafts before the gas was run in. They stood high and hemispherical, like dewdrops; and after various periods were

removed. It was possible to do this with no other alteration of conditions within the chamber than were involved by the introduction from above of a long-handled forceps carrying a piece of filter paper to blot up the fluid. At times when the injured graft was becoming acid in consequence of the exposure to CO_2 but had not yet attained to the hue of *orange* a narrow circle of this hue could be briefly seen after the application of the filter paper, a circle corresponding in situation to the edge of the drop, the place at which the layer of water had been thinnest. When now the graft was reexposed to air the regions still moist turned purple first. Healthy grafts gave less outspoken findings; and only occasionally were slight differences of the general nature of those just described to be seen on the intact skin.

The experiments brought out a number of facts. Carbon dioxide penetrates the intact skin of the shaved mouse so rapidly as to cause some change in the surface hue of animals stained with vital red; it renders vigorous skin grafts somewhat more acid than they already are as the result of ischemia; and it penetrates injured grafts with an astonishing rapidity, rendering them pronouncedly acid. How acid the injured tissue can become is not yet certain, for no indicator other than phenol red has been employed.

Even in vigorous skin grafts there is much cell degeneration and death. The epithelium in particular is soon reduced to a thin layer of living cells (21). When the tissue has been injured experimentally the retrograde changes must be still greater; and one might think of the heated, or frozen and thawed, graft as a mere raw dead surface exposed to the air, did not its texture and the absence of seepage or drying belie the view. Doubtless the alkaline reaction of grafts thus injured is due in some part to an escape of carbon dioxide from them. The experiments involving exposure for a long time to a gas mixture containing 10.35 per cent CO_2 possess significance in this connection. Exposure to such a proportion of CO_2 , approximately twice that in alveolar air, leads to an alteration in the hue of injured grafts,—there is a change from *eugenia red* (or purpler) to *carrot red*; but the alteration is slight, as compared with the change to *apricot orange* promptly undergone by stained pieces of healthy skin when grafted in an atmosphere of air. Exposure to 10.35 per cent CO_2 does not result in any definite color change in the mouse's body surface generally, and leads to only dubious ones in healthy grafts already *in situ*.

The Association of Tissue Acidosis and Edema.

Healthy skin grafts are regularly somewhat edematous during the first few days after implantation, at the time that is to say when the local reaction is acid as compared with that of the rest of the integument. A similar association of edema with tissue acidosis is not infrequent under other circumstances,—prevailing opinion and potentiometric determinations to the contrary notwithstanding. It can be observed to exist about surface abrasions in animals vitally stained with phenol red, as well as at other points of local inflammation, the phthalein in the swollen areas being orange as compared with the red of that in the normal skin nearby. And a rapid local development of edema and acidosis takes place when a tube through which water circulates at 50–52°C. is applied to the skin of an anesthetized rat stained with phenol red. Under such circumstances the edema and a change from red to orange of the phthalein contained in the region involved by it both become pronounced within 15 minutes.

In contrast to the swelling and acidity manifested by healthy grafts one sees in frozen and thawed grafts not the least edema, and a reaction which is definitely alkaline. The facts are not to be taken, however, as furnishing support to the hypothesis that acidosis determines edema. Edema develops, yes, in the living and acid skin graft, but it progressively disappears during the days immediately after the grafting, whereas the acidosis does not diminish. Furthermore edema is regularly to be met in grafts that have been injured by heat, although the reaction of the injured tissue is pronouncedly alkaline. In a succeeding paper observations are reported which would suggest that under the conditions of widespread and enduring tissue acidosis brought about with hydrochloric acid no important water retention occurs.

DISCUSSION.

The foregoing observations provide data on the conditions which prevail in skin grafts and determine their survival; but it is in a broader relation that they have principal claim to attention,—namely, in relation to the happenings within ischemic tissues as a class. Despite the general recognition that interference with the blood supply

of a living tissue results in the local formation of acid substances the corollary that ischemic tissues must endure for some time in an acid milieu if they are to survive seems not to have had the attention that it merits. As the present work shows, the more vigorous the cells the more acid do they render the tissue when its blood supply is interfered with; and the tissue survives despite this acidity. Skin has a relatively slight metabolic activity, and some part of the carbon dioxide accumulating in avascular grafts of it must escape to the air or into the body of the animal, while a continuous, if slow, fluid interchange with the neighboring tissues acts also to reduce the local acidity. Yet notably acid the tissue is, nevertheless. This being the case what must one suppose the reaction to be within a leg severed and reunited without suture of the vessels (Halsted), or in an arm surviving despite a clot in the axillary artery? Can one doubt the development of a more pronounced acidity in these ischemic members?

That cells of some sorts will survive and proliferate *in vitro* in a frankly acid medium is a fact sufficiently attested (22). But the occurrence of proliferation renders the case somewhat different from that of tissue surviving within the body under acid conditions. For it might well be that the individual elements of a culture tolerate the condition of acidity but poorly, and that the strain survives only because its elements continue to divide, furnishing fresh entities more rapidly than the injurious medium kills them off. An analogy to this state of affairs is to be found in the growth of certain tumors composed of cells surviving for so short a time that retrogression of the mass would inevitably ensue were not the rate of wastage more than outstripped by that of proliferation. The condition is one familiar to every student of neoplasms. It is often plainly evident when "Chicken Tumor I,"—a transmissible sarcoma,—is cultivated *in vitro*.

No attempt has been made in the present work to determine more than approximately the degree of acidity developing in the grafted skin of the mouse. Manifestly the knowledge could have only a special interest; for one would expect a much greater acidity to develop in tissues of high metabolic activity. To determine the relative reaction within ischemic portions, living and dead, of organs which appear to be frankly acid under normal conditions,—the liver and pancreas for example,—will be an interesting task for the future.

Current generalizations on the changes which take place within tissues dying in the body are largely based upon studies of material

autolyzing in the mass *in vitro*; and perhaps they can be safely applied to large masses autolyzing *in vivo*. The interchange of material with the body round about will have little significance for the immediate fate of large infarcts and accumulations of pus despite the unusual permeability of dead tissue. But when the necrotic mass is small the factor of interchange assumes great importance, as the experiments with the skin grafts show. The occurrences subsequent to the injury of small cell aggregates cannot be explained on the basis that a local accumulation of acids determines autolysis or atrophy (23), for the sufficient reason that the local reaction will tend toward alkalinity rather than toward the acidity of vigorous tissue surviving an ischemia. It follows that the chemical changes which take place in small necroses must differ in some respects from those occurring in large masses of dead tissue. A single set of generalizations as concerns autolytic processes will not cover both instances.

While the life processes of vigorous tissue suddenly rendered bloodless act to create a milieu that would seem prejudicial to survival, the alterations that take place in injured tissue would appear superficially to favor this event. To judge from the observations on skin the lessened cell activity consequent upon injury results in a smaller accumulation of acid material,—save for that referable to the trauma itself (as in muscle); and the increased permeability of the damaged tissue results in a more rapid escape from it of carbon dioxide and of the other substances causing acidity. But needless to say the rough correspondence thus brought about between the reaction of the injured graft and the normal tissue surrounding it is a superficial phenomenon, not the sign of a good state of affairs but of one which masks profound cytological derangements.

The eventual drying of grafts which fail to “take” is not due to any failure to obtain fluid from underneath, as the experiments have shown. Rather must one think of it as consequent on an abnormal loss of fluid from the surface, itself a manifestation of the increased permeability which can be demonstrated by exposure of the tissue to carbon dioxide. There is good reason to suppose that the reaction of the tissue involved in skin lesions often deviates from the normal owing to the influence of the factors dealt with in the present work.

The observation that edema and tissue acidosis sometimes coexist

would at first sight seem difficult to reconcile with the fact that edema fluids as obtained for potentiometric examination are regularly alkaline (24). But the contradiction is merely apparent. Local acidosis and edema occur together only when the fluid accumulation is not very pronounced and the metabolic activities of the tissue are either abnormally heightened,—as during inflammation,—or are taking place under conditions which permit of an accumulation of acid products,—as in skin grafts. There is little doubt that the development of edema can act to maintain alkalinity, the profuse alkaline fluid deriving from the blood having effect to drown out, so to speak, what might otherwise be a local acidosis. I have never found a very pronounced inflammatory edema that did not yield a fluid alkaline to phenol red, although inflammation as such conduces to local acidosis. And Henning (25) who injected $N/10$ HCl into the leg muscles of guinea pigs, observed that the initial acidity was supplanted after 24 hours by a pronounced local edema and alkalinity. It is conceivable that sometimes during the development of an inflammatory edema there may be such an escape of blood protein into the tissues as will suffice to influence phenol red, with result in a greater alteration in the color of the phthalein than the actual acidosis would warrant. No such happening can be invoked to explain the case of edematous skin grafts, however.

Some comment is necessary on the difference between the hue of the skin surface of the vitally stained mouse, as seen by reflected light, and the color of the corium—the skin component principally stained with phenol red—when viewed by transmitted light. The purply red of the former corresponds with pH 7.5 to 7.6, as ascertained by the wedge method described in the present paper; whereas the connective tissue of the corium, when examined separately under oil, has the yellow-pink of about pH 7.2 (26). The reasons for this difference—one which persists when the blood vessels have been flushed out—are largely to be found in the differing optical conditions, but also in some part in the suffusion of the tissue with an alkaline lymph, heavily charged with phthalein, and in consequence ruddy. When a flap of the oiled skin surface of a deeply stained mouse is compressed between slides so that the interstitial fluid is forced out of it for a moment its color is altered from yellowish red to orange.

The observations here set forth may, perhaps, be thought of as the first steps in an analysis of the physical factors which act to determine the fate of engrafted tissues. It is habit to suppose that this fate depends on the ability to survive temporary ischemia, on absence of infection, prompt vascularization and, in the case of iso-grafts, on a tolerance by the host of the strange tissue, and by the graft of its alien surroundings. So of course it does. But the first requisite for survival, namely the ability to survive ischemia is directly referable to physical conditions within the graft, as is also, doubtless, the development of the vasculature that will eventually rescue the tissue from its precarious state.

Gesell has recently brought forward (27) a theory of respiratory control based on the assumption that changes in the hydrogen ion concentration of the respiratory center are the responsible influence rather than changes within the blood. The happenings within skin grafts furnish a suggestive analogue to what goes on within the center, according to the view of this author.

SUMMARY.

By means of vital staining with indicators a study has been made of the changes in reaction and in certain other attributes of a tissue abruptly rendered ischemic. Grafts of mouse skin have been employed as test material. It has been found that almost at once after implantation vigorous grafts become notably acid as compared with the normal skin and that they survive and "take" despite the acid condition, which remains at a maximum for several days. Weak or injured grafts on the contrary tend to be as alkaline as their surroundings, if not more so. Through experiments directed to the purpose reasons for this difference have been found in the lessened metabolic activities of the cells of the injured skin, and in an increased permeability which leads to a generalized suffusion of the damaged tissue with the alkaline lymph and an abnormally rapid escape of carbon dioxide from it. The influence of these factors to determine the reaction of tissues dying within the body has not been sufficiently taken into account in considering the chemical changes that occur after cell death, and some revision of current views regarding these as they affect small necrotic masses would seem called for.

Carbon dioxide penetrates so readily into the living skin as to cause some local increase in the hydrogen ion concentration within cutaneous regions exposed to an atmosphere of it, even when the local circulation and the ventilation by way of the lungs have not been interfered with. It penetrates injured skin with especially great ease.

Tissue acidosis and edema not infrequently occur together; but no relationship between them of cause and effect has been made out.

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EXPLANATION OF PLATE 32.

FIGS. 1, 2, and 3. Right and left sides of a mouse, to show the course of the vital staining of grafts with phenol red. Period: any time during the first 3 days after implantation.

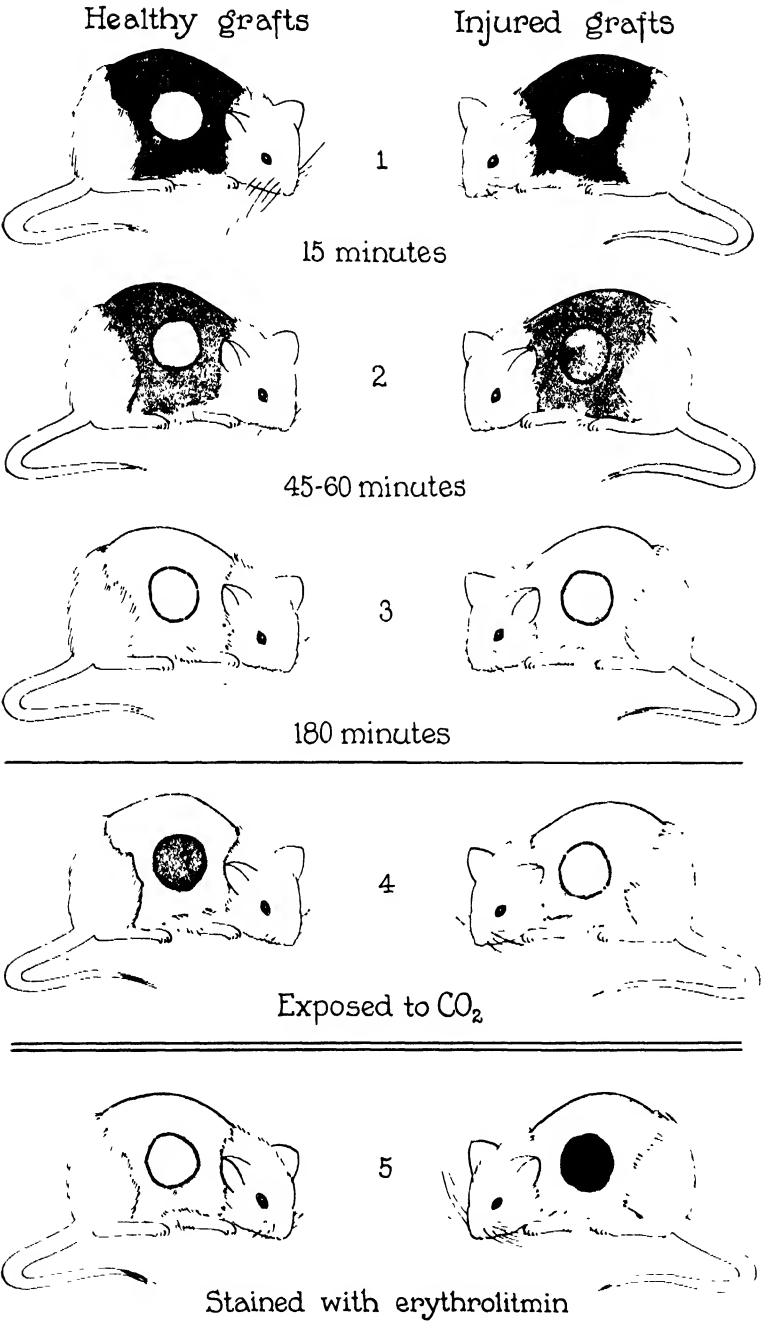
FIG. 1. The staining 15 minutes after an intraperitoneal injection of the indicator. The healthy graft shows no color as yet, whereas the one that was frozen and thawed has become pink. The animal is already deeply colored.

FIG. 2. 45 to 60 minutes after the injection. The grafts are now intensely stained, the hue of the healthy one being indicative of a condition of relative acidity, whereas that which has been injured would seem to be slightly more alkaline than the normal tissue round about, judging from its color. The mouse itself is beginning to decolorize.

FIG. 3. 3 hours after the injection. The animal is now completely decolorized but there is still much phenol red within the grafts and the same differences are visible in them as before.

FIG. 4. Effect on the phenol red coloration of exposure of the body surface to an atmosphere of carbon dioxide for 15 minutes. The injured graft is now no longer alkaline but frankly acid, as evidenced by its hue; and the healthy graft while slightly more acid than ordinary is not as acid as the injured graft. The color of the body surface generally is indicative of a slight change in the direction of acidity.

FIG. 5. Right and left sides of a mouse stained with erythrolitmin, to show the alterations in color of engrafted skin and of skin that has been purposely damaged as well as engrafted. The healthy graft is slightly bluer than the body surface generally, a fact which attests to some injury to its cells with a loss of the normal semipermeability; and the graft which was frozen and thawed is deep blue,—evidence that the cells have become freely permeable to the alkaline body fluids.



THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VII. THE INFLUENCE OF CHANGES IN THE REACTION OF THE BLOOD UPON THE REACTION OF THE TISSUES.

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The blood is all things to all tissues, and in consequence it can scarcely be expected to manifest the peculiarities of any one of them. Special characters of its own it must have to serve so widely as it does. These elementary principles are often overlooked by those who would speculate upon hydrogen ion concentrations within the body; and one finds not infrequent a disposition to suppose that the tissues and blood must have the same reaction. In several previous papers from this laboratory evidence has been produced that the hydrogen ion concentration within the individual organs differs somewhat (1). In this respect the tissues would appear to be related to the blood as to a sort of physiological base line, from which they stand off at various distances along the ordinate of hydrogen ion concentration. When one produces alterations in the level of the base line, as in the experiments now to be described, the position of many tissues along the ordinate alters also; they become relatively more or less acid or alkaline in rough correspondence with what happens in the blood. Other tissues however, as we shall show, retain their apparent pH unchanged even when the amount of acid or alkali introduced into the animal is sufficient to cause death.

The accumulated data with regard to the various offscourings of the organs as they appear in the blood and the excreta have furnished material for many inferences upon tissue states. But these have remained inferences at best, subject to modification with each new parcel of facts discovered. There have been few attempts to study the reaction of the tissues directly save by quantitative methods

the practice of which involves distortion from the conditions of life. Mention should be made, however, of the interesting experiments of Stieglitz on the reaction of the living kidney as affected by the administration of acid or alkali (2).

Method.

For the present work rats vitally stained with phthaleins were employed; and the living tissues were examined under circumstances which would seem to exclude the ordinary errors of interpretation.

A pair of male rats, from the same stock and of the same age and weight, preferably not full grown, were used in each experiment. They were given no food on the morning of it. The hair was removed from the chest, abdomen, and sides a day or two beforehand in order that any inflammation due to the shaving might have cleared up. On beginning the experiment each animal was given 20 per cent urethane (3) subcutaneously into the back of the neck, in the proportion of 1.1 cc. for every 150 gm. weight, and some minutes later the phthalein into the peritoneal cavity. After $\frac{1}{2}$ hour or more, when the general staining was pronounced, the animals, by now unconscious, were submerged to the muzzle in warm paraffin oil in glass dishes placed side by side under identical conditions of lighting, and after brief preliminary observations the injection of acid or alkali into a jugular vein was begun.

The rectangular Pyrex dishes had a layer of solid paraffin covering the bottom, with a depression for the urine, and they held sufficient liquid paraffin oil—cleansed, and inert to phthalein—to cover the rats. The oil was kept warm by the heat generated when a current was run through coiled “Nichrome” wires surrounded by oil and encased in glass tubing bent to conform to the shape of the dish (Fig. 1). The heating was regulated by means of a rheostat. To distribute it evenly the oil was stirred at intervals with a spatula. The temperature in the two dishes was maintained between 38° and 41°, as nearly the same in both as possible. The legs of the rats were kept in extension by means of long pins thrust through the skin of the paws and into the paraffin bed, while their muzzles were held above the surface of the oil by a thread carried through the skin of the nose and tied about a knitting needle placed across the dish from side to side. Before the oil was poured on, a fine cannula was inserted into a jugular vein of one of the animals, and connected with the apparatus for the injection of the acid or alkaline solution. The other animal served as control. A special arrangement was devised

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- (3) The influence of urethane upon the blood reaction of rats is known, thanks to the work of Hawkins (Hawkins, J. A., and Murphy, Jas. B., *J. Exp. Med.*, 1925, xlii, 609). For 3 hours or more after injection it causes little change but then there ensues an alkalosis. Our experiments were completed within the initial 3 hours.

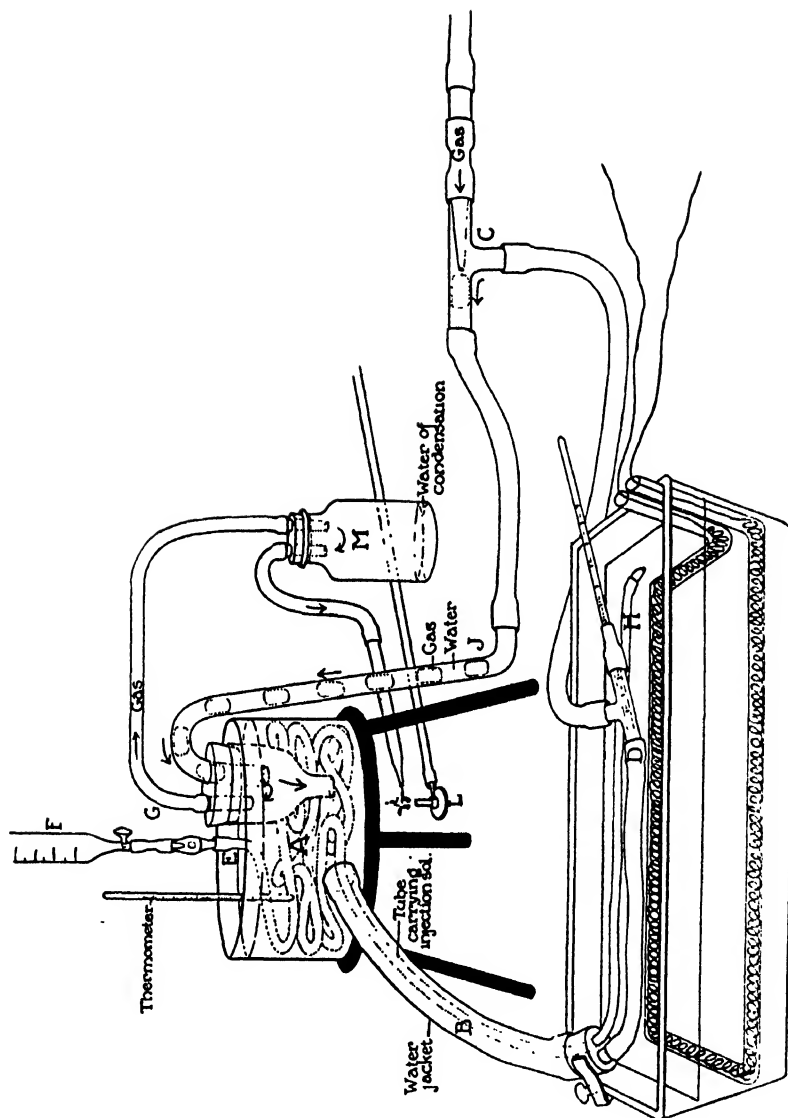


FIG. 1.

to deliver the injection fluid at body temperature (Fig. 1). The nozzle of a calibrated burette (*F*) which served as a reservoir was connected with a drop gauge (*E*) and this in turn with a long rubber tube coiled in a bath containing hot water at a constant temperature (*A*). The tube passed out through a side arm from the bath, and was encased, as far as the oil bath, in a much larger tube (*B*) containing some of the same warmed water that was in the bath. In order to ensure a circulation in this elongate jacket its further, stoppered end was equipped with an exit pipe (*D*) for the water, in addition to the opening for the rubber tube carrying the injection fluid (*H*). The water was kept flowing through the jacket and back into the bath by the introduction of a slow stream of illuminating gas through a side arm (*C*) of the exit pipe. The passage of bubbles of gas along the pipe (*J*) acted to move water back to the water bath. The gas was delivered separately to be burnt beneath this latter. The water of condensation from the gas collected in the bottle (*M*). For the idea embodied in the apparatus we are indebted to Dr. D. R. Drury.

The tube carrying the solution to be injected now passed beneath the surface of the oil and laterally along the dish,—where its temperature was measured with a thermometer let into the system,—and so to the animal. Near the cannula there was a side arm,—which is not figured,—to permit an adjustment of the pressure immediately after connection with the jugular had been made. Otherwise, when the clamp was taken from the vessel prior to the injection there was sometimes a sudden gush of fluid into the blood with death at once. To prevent blood from passing back into the cannula the latter was turned at an angle to the course of the vein so that its wall would act as a valve across the opening in the glass.

The intensity of the staining of paired rats injected with an equal dose of phthalein was frequently found to differ somewhat; and when phenol red had been used the surface hue often differed slightly as well, variations from *eugenia red* to *jasper red* (4) being observed. The differences in extravascular pH, indicated by these differences in hue, are no greater than the long recognized individual differences in pH of the normal blood. In order to minimize such errors as might be introduced by them when the tissues of the injected and the control rat were compared, several individuals of the same size were often given phenol red at one time and from these the two were selected that most closely corresponded in the character of the staining.

As many of the tissues were examined *in situ* as possible. Since skilled assistance was available the examinations could be made very rapidly. The color of the body surface generally, and of the paws, ears, and mucous membranes, gave

(4) *vide* Ridgway, R., Color standards and nomenclature, Published by the Author, Washington, D. C., 1912. Our method of utilizing this book has been described in a previous paper (Rous, P., *J. Exp. Med.*, 1926, xliv, 815).

direct indications of the reaction of lymph, connective tissue, and cartilage. By a bloodless incision the tendon could be exposed, as too the lymph node of the groin, and the voluntary muscle and the cartilages of the thorax. But the colors in general were most truly seen when the tissues were viewed by transmitted light. This was possible without disturbance, in the case of the connective tissue of a skin fold, and of the cartilage of the ear. Small bits of the other organs were snipped off, and, still surrounded by oil, were examined between mica slips, those from the control and the experimental animal being studied side by side. Glass slides could not be used because of the alkali deriving from them (5). A special plan was followed in taking the specimens to rule out the possibility that the differences noted were due to supravital changes. Thus, when an animal had been injected with acid the bit from it was removed for inspection only after that from the control had been secured,—since the bit longest out of the body would tend to be the more acid as result of the longer asphyxia from interruption of the circulation. If now under these circumstances the tissue fragment just removed from the injected rat appeared more acid than that from the control there was good reason to suppose that a real difference in reaction had existed within the body. Sometimes, to obtain slices of the parenchymal organs, Valentine knives were employed, a different one for each animal, and the cutting was done under oil, on a paraffin base. Bleeding from severed vessels was prevented with mosquito forceps; and cleansed and dried instruments were employed with each specimen. Special care was necessary to avoid contamination with the blood, which, having received directly a greater or less quantity of the acid or alkaline solution, was, of course, altered thereby. In the cases of the parenchymal organs such contamination inevitably occurred, but fortunately it was the tissues of just these organs that remained uninfluenced in reaction by the injections. Cartilage, connective tissue, and tendon could readily be obtained blood-free. It is possible to procure preparations containing all three of these tissues and bone as well from the tip of the tail of the young animal (65 to 90 gm.). This is snipped off with scissors, gently pressed between folds of washed gauze to drive out the blood, and the bony core is rapidly extracted under oil and mounted between mica slides for inspection by transmitted light. By properly timing the procedures one can compare the experimental specimen directly with that from a control treated in precisely the same way and after the same brief interval of separation from the body. Bone and voluntary muscle were not satisfactorily stained under the circumstances of the experiments,—which usually involved a considerable diuresis with color loss,—nor was the epidermis stained nor the splenic substance. A study of the stomach, intestines, and lungs was not made. To all practical purposes, then, the observations were limited to the superficial color, to the hue of the tissue of the ears, *in situ* or on dissection, to connective tissue and cartilage as such, tendon, liver, pancreas, kidney, and superficial lymph nodes. When dissolved in fat the phthaleins no longer act as indicators, though they do in the adipose tissue itself.

The phthaleins employed were cresol red, phenol red, and brom cresol purple. They were employed in watery solution isotonic with the blood and at pH 7.4 except where otherwise stated. The staining was at a maximum when the injection of acid or alkali was begun, practically all the phthalein having been absorbed from the peritoneal cavity. Brom thymol blue could not be used since it is toxic and gives false indications *in vivo* (6). Nothing has been found to date despite much search in our laboratory that will take its place in covering the zone between the ranges of phenol red and brom cresol purple,—a zone in which the reaction of many of the tissues would seem to lie.

Changes in the Coloration Result from the Injections.

In some preliminary observations, to determine the general effects of acid and alkali upon the hues of the stained tissues, relatively concentrated solutions of sodium carbonate and of hydrochloric acid were run into the blood stream.

These had the strengths employed by MacNider (7) in his studies upon kidney function, that is to say they were equimolecular with 1.5 per cent NaCl and, in the case of carbonate, 3 per cent NaCl (concentrations slightly less than $N/2$ and $N/1$); and hence they were notably hypertonic. To lessen the likelihood of anhydremia from this cause, with "outlying acidosis" as a sequel (8), several cc. of warm water was given to the animal by stomach tube some minutes prior to the injection. The acid or alkali was run in as fast as possible, which was after all rather slowly. Tests showed that about 4 cc. of $N/1$ Na_2CO_3 could be introduced into a rat of 170 gm. in the course of an hour, with the animal still in excellent condition, but if the rate was increased a very little, or a few drops were inadvertently run in together after the injection had proceeded for some time death occurred immediately. Since $N/2$ HCl greatly damages the red cells $N/4$ HCl was early substituted for it. More than 5 cc. of the latter solution, given during the course of half an hour, was tolerated by a rat of 140 gm. The patching characteristic of an "outlying acidosis" was never noticed.

Shortly after the administration of acid was begun the secretion of urine stopped, as in the case of MacNider's animals; whereas that of the alkali was attended by a profuse diuresis. The injections were pushed to the lethal point, but from time to time while the condition of the animal was still good, the color of bits of its vitally stained organs were compared with those from the control by the technic above described. It was soon found that there exists a group of tissues,—connective tissue, tendons, fascia, and cartilage,—which undergo marked alterations in hydrogen ion concentration, as indi-

cated by the phthaleins, when acid or alkali is given. The hue of certain other tissues, parenchymal in make-up,—those of the liver, pancreas, and lymph nodes,—remains practically unchanged so long as life lasts. The tissue which responds soonest to the alteration in the reaction of blood is the connective tissue.

Saline Solution Fails to Influence the Tissue Reaction.

More extensive experiments were now undertaken with solutions isotonic with the blood (N/6.35 solutions). Large amounts, 15 cc. or more, were tolerated by rats of 150 gm.; and there was diuresis after the acid as well as after alkali. The question arose of how far the observed alterations in the tissue colors were referable to the amount of fluid introduced, merely as such. To control this factor some injections were made of 0.92 per cent NaCl. It was found that 20 cc., run into 150 gm. rats at the rate of the acid or alkali, caused not the slightest change in the hue of the tissues stained with phenol red except the fading incident to rapid elimination of the indicator. A like result followed the injection into a 110 gm. rat of 15 cc. of isotonic dextrose solution (5.4 per cent Merck's dextrose in twice distilled water).

The findings with hypertonic solutions of acid and alkali did not differ essentially from those with the isotonic solutions save that in the case of the former the phthalein was retained longer in the body; so it will be unnecessary to discriminate between the two sets of experiments in detailing the results.

Effects of Alkali on the Coloration with Cresol Red.

The surface hue of a living rabbit stained with phenol red and viewed under oil by reflected light is indicative of a reaction between pH 7.4 and pH 7.6, as can be shown by comparing it, through an Autenrieth wedge containing water, with the hue obtained by placing on the surface of a shaved but unstained control, one or another of a series of similar wedges filled with buffer solutions of appropriate pH, stained with phenol red. The surface color of urethanized and stained rats is indicative of the same slight degree of alkalinity. The subcutaneous tissue as such appears relatively acid by transmitted light,

having the orange-pink of an apparent pH of about 7.2; and the more ruddy surface hue is to be ascribed, in part to the optical conditions and in other part to a staining of the alkaline interstitial fluids (9). When cresol red has been injected the oiled surface of the stained rat appears yellow, slightly tinged with brown, and all the stained tissues are a brilliant yellow. Several experiments were made to determine whether the color could not be altered to the rose-purple which is characteristic of a pH of 7.8 or more, under controlled conditions.

In an initial test a Na_2CO_3 solution isotonic with 1.5 per cent NaCl was gradually injected into a rat of 110 gm. The animal died when 9.0 cc. had been run in. The urine was orange-red to begin with but later purple red; yet none of the stained organs changed from the usual yellow hue. In the course of further observations, with N/1 sodium carbonate, the blood changed from orange-red toward purple, and the surface color from yellow to rose-pink, after 3.7 cc. of the solution had been run into an animal of 170 gm. stained by the injection of 1.7 cc. of 4 per cent cresol red solution. The nose, lips, and ears became light red; yet the tissues generally appeared of the same clear yellow as in the control, when the blood and intercellular fluid were rapidly pressed from them. A rat of 250 gm. given 10.4 cc. of N/6.35 sodium carbonate became ruddy orange, and its urine much more purple than that of the control. The ears *in situ* appeared ruddy and so too did a skin flap dissected up and viewed by transmitted light. But a wedge-shaped piece of the ear, rapidly excised and compressed to drive out the fluid, was as yellow as the ear of the control; and so too with all of the other tissues examined, connective tissue, cartilage, tendon, voluntary muscle, liver, lymph nodes, kidney, and pancreas. Many of these looked somewhat ruddy until pressed upon to expel the stained fluid associated with them.

It was obvious from these experiments that only the blood and intercellular fluids could be altered sufficiently in reaction by the injection of alkali to come within the range of cresol red. This would have meant a considerable change, granting,—what has not yet been proven,—that cresol red when in the body serves to indicate the pH as accurately as it does *in vitro*. For a rose-purple hue of the tissues could scarcely be expected short of pH 7.8 or 8.0; and the most alkaline of them, the connective tissue, exhibits under ordinary circumstances when stained with phenol red,—an indicator well tested in this relation (10),—the hue indicative of an approximate pH of 7.2.

Effects of Alkali on the Coloration with Phenol Red.

The color of the shaved and oiled body surface of healthy animals injected with phenol red and viewed by reflected light corresponds, as has been stated, to that associated with a pH of 7.4 to 7.6 under controlled conditions. Many of the organs, notably the parenchymal ones, are stained clear yellow, indicating that their reaction lies beyond the range of the phthalein on the acid side; but two of the matrix tissues,—the connective tissue and tendon,—exhibit shades of pinky yellow which point to the presence of a relative alkalinity within them, the connective tissue appearing to be at about pH 7.2, and the tendons at, or slightly above, pH 7.0 (11). If the introduction of alkali into the blood alters the reaction of these two tissues one would expect the change to be demonstrable with phenol red since their apparent normal reaction lies well within the range of this phthalein. Such is the actual case.

In preliminary experiments hypertonic solutions of sodium carbonate were employed. Shortly after the alkali had begun to run in the rats were noted to be purpler than the controls, and soon the general hue was a brilliant rose-purple, especially pronounced on the nose, mucous membranes, and ears. The blood coursing through the latter was seen to be intensely purple as compared with red in the controls. The urine, now voided in great quantity, was no longer stained red but purple. When the alkalosis had become pronounced examination showed that both the connective tissue and tendons had altered from pinky yellow to old rose; and the cartilage was affected as well, being now ruddy orange instead of the usual orange-yellow. Some young animals of 50 to 80 gm., in which the injection was pushed to the limit compatible with life, had flat bones of the skull that appeared pinker in the growing portions than those of the controls: but owing to difficulties of the dissection this difference cannot be stressed. The differences observed in tissues of the "core" of the tails of young animals were clear-cut, and leave no doubt that the reaction of the connective tissue, tendon, and cartilage had been altered *in vivo*. The brief ischemia incident to the examination could not have killed these hardy matrix tissues, of course. Wedges cut rapidly from the ears and pressed to force out the ruddy interstitial fluid were orange in the case of the injected animal, yellow in the control, as would follow from the fact that they consisted mainly of cartilage.

The pancreas, liver, lymph nodes, and kidney cortex were always of the same brilliant yellow as in the control even when the animal was moribund from the large amount of alkali injected. In one case the Malpighian bodies of the spleen were also observed to be yellow. The peripheral nerves were unstained but

the hue of their sheaths was changed from pinky yellow to old rose,—which one would expect from their character as connective tissue; and the walls of the large arteries were old rose. The lymph was much purpler than in the controls.

The changes observed in animals given strong alkali were duplicated when it was administered in N/6.35 solution. The isotonic carbonate solution was especially well tolerated, and we have injected 6 cc. into an animal of 65 gm. during the course of 1½ hours without causing death, and 27½ cc. into a 200 gm. animal in about the same time. Whenever there was an initial color difference in the pair of rats we regularly injected that individual which had the lighter, and sometimes the less alkaline, hue,—*jasper red* for example as compared with *eugenia red*. The changes brought about by the alkali were the more remarkable under such circumstances. As the injection progressed one saw the rat which was originally the lighter with perhaps a slightly yellow cast to the surface red, become relatively dark and purple, and those of its tissues turning toward purple appeared more markedly stained than was the case in the control, although they really had less of the phthalein in them—a fact demonstrated by applying alkali to bits of both *in vitro*. The tissues of the injected animal which did not alter from the ordinary hue, remaining yellow, were a paler yellow than those of the control because of a more rapid loss of phthalein by elimination of it from the body.

Situation of the Phthaleins.

Changes in the color of the blood can be ruled out as a cause for the alterations in the tissue hue, save in the case of especially vascular organs (ears, nose, mouth, and tongue) viewed *in situ*; for the prevailing color on staining with phenol red and other phthaleins is due to an extravascular penetration of the dye (12). It might be urged however that changes in the reaction of the interstitial fluids, secondary to those taking place in the blood, will suffice to explain the observed color phenomena. This is not the case. True, the suffusion of the matrix tissues with purple interstitial fluid tends to intensify their color. But the phthalein has a tendency to become fixed on the tissue components themselves (13). A bit of connective tissue or tendon removed from a rat stained *in vivo*, frayed with the knife, and examined under the high power, will regularly show a brilliant homogeneous staining of even its finest filaments. So too with the thinnest edge of a sliver of cartilage. The cells of such organs as stain yellow with phenol red, as if acid, are yellow even after alkali has been administered despite the presence of a purple red interstitial fluid, that is to say one which is relatively alkaline. Whether the cells of the matrix tissues as well as the ground substance become stained is not certain. It is safest certainly, for the present, to look upon the phthalein findings as expressive of the reaction within tissues, as distinct from that within cells (14).

The results obtained with phenol red were consistent. They showed not only that altering the reaction of the blood toward alkalinity

resulted in changes in the reaction of some of the tissues, but that the ones evidently affected were those of which this might have been predicted owing to the ease with which they are penetrated by substances escaping from the blood stream (15) and to the further circumstance that ordinarily their apparent reaction, as indicated by the color they assume with phenol red, lies well within the range of this phthalein. Of the tissues with reactions lying beyond the range of phenol red,—on the acid side of it,—only one was so influenced by the alkali as to alter in hue. This tissue, the cartilage, would appear to be only slightly more acid under ordinary conditions than are connective tissue and tendon, occupying in this respect a middle place between them and the parenchymal organs (16). For all that is known to the contrary the reaction of these latter organs might have been changed by the alkali without bringing them within the range of phenol red. To test for such an occurrence, as well as to obtain a check on the general findings, brom cresol purple was now employed.

Effects of Alkali on the Coloration with Brom Cresol Purple.

Brom cresol purple is not stable in a 4 per cent solution brought to pH 7.4 but undergoes a rapid chemical change (17) which leaves it something of an indicator still but with a different range and inferior color values. The deterioration can be prevented in 1 per cent solutions by a special mode of preparation (18), but at 4 per cent it begins within a few minutes at most after the titration, as a change from purple to orange, starting at one point as a rule and rapidly spreading throughout the fluid. It does not take place when there has been a slight overalkalinization, and resort was had to this procedure in order to obtain stable preparations for injection. 400 mg. of brom cresol purple was taken up in 1.6 cc. of N/1 NaOH and diluted to 10 cc. with distilled water. Had 1.48 N/1 been used instead the solution would have been at pH 7.4. The rats tolerated well the intraperitoneal injection of the phthalein treated thus, when given in the proportion of 1.6 cc. for every 150 gm. of animal. Test observations on the color of the indicator diffusing into buffer solutions from pieces of the vitally stained tissues proved that it had undergone none of the deterioration just described.

The rats stained with brom cresol purple and injected with sodium carbonate, either in isotonic or hypertonic solution (54.6 gm. to a liter, equimolecular that is to say with 3 per cent NaCl), showed striking alterations in the surface hue. They became much more purple, *dull blue-violet*, in Ridgway's nomenclature, as compared with the *dull violet-blue* of uninjected animals. The phenomenon could not be accounted for by a retention of the dye in the tissues at a time when the controls had begun to decolorize, since no such retention occurred but on the

contrary a greater elimination by the experimental animals. The alkali injection regularly resulted in profuse diuresis, with a purple urine; but the urine of the controls was purple also, that is to say alkaline, probably because of the slight excess of alkali introduced with the indicator. By injecting the control animals with sodium carbonate, at a time when they had come to appear much less purple than the experimental ones they could be brought to the same, or a deeper hue.

The blood and lymph (as pressed from the superficial lymph nodes) both appeared blue-purple prior to any administration of carbonate; and they did not become more purple after it. The fact carries implications of importance. When really at pH 7.4 to 7.5,—the reaction of rat blood (19),—brom cresol purple is so largely dissociated that further alkalinization fails to result in an increase in the purple hue appreciable to the naked eye under test-tube conditions. It follows that no change in the color of blood stained with the phthalein should occur upon alkalinization *in vivo* if the circulating indicator has still the range characterizing it under controlled conditions *in vitro*. And no change does occur either in blood or lymph. Yet there is an alteration in the color of the body surface, which becomes more purple. This can only be attributed to an alteration in the hue of the subcutaneous tissues,—the actual event as direct inspection shows. These tissues, less alkaline than the blood, as judged by their hue with phenol red, must be less alkaline too with brom cresol purple; for otherwise the injection of sodium carbonate would not cause their color to intensify. The results with the two indicators are consistent in the demonstration of this difference in relative reaction.

Not only does the connective tissue of the animal injected with carbonate become more purple but so too do the tendons and cartilage. When a segment of the ear, which consists mainly of the last of the tissues mentioned, is excised and compressed between mica surfaces it can be seen to be far more purple than the control segment; whereas the almost blood-free fluid extruded from it and protected by oil has the same hue in both cases. The core of the tip of the tail of the injected rat also shows a more purple cartilage, and the costochondral articulations are a deeper purple. The bones stain poorly, and no satisfactory color comparison is to be had with them. In contrast to the ease with which the hue of most of the matrix tissues is altered that of the parenchymal organs evinces no change. The blue-green or yellow-green color of the lymph nodes, liver, and pancreas remains the same even when so much alkali had been introduced that the animal is in a moribund condition.

Valentine knife sections of the kidney cortex of our alkalinized animals were purple to the eye as compared with the yellow-green of the control tissues; and under the microscope one could see that the tubules contained a far more intensely purple urine and that a great many of them were colored purply blue. The differences will not be gone into however since they were the product of especially complicated conditions. An account of them will be reserved for a later paper from this laboratory.

These observations concluded the series on the effects of injected alkali on the coloration of tissues stained with phthaleins. The results of the various experiments were in complete accord in showing that there exists a group of tissues—matrix tissues—which become more alkaline when the blood is alkalinized; and a second group of tissues, parenchymal in constitution, which are to all appearance unaffected even when alkalinization is pushed to the extreme. Tests were now begun upon the effects of acid.

Effects of Acid on the Coloration with Phenol Red.

There are few tissues staining with phenol red of which any color alteration can be expected on the injection of acid; for only a few have internal conditions bringing them within the range of this indicator, the reaction of the others appearing to lie beyond it, on the acid side (20). The connective tissue and tendons stain yellow-pink and pinky yellow respectively; and these colors might conceivably be altered to a frank orange-yellow when acid is administered. This has proved to be the case.

When N/6.35 HCl was gradually run into a jugular vein of the stained rat the body surface, which had been *jasper red*, became *coral red*, then *carnelian red* and eventually *rufous*. Meantime the connective tissue turned from salmon to orange-yellow, and the tendon, which had not been so pink to begin with, took on the same hue. The blood and lymph became somewhat less rosy than usual. The other tissues, being ordinarily orange-yellow, did not alter in hue. There was some diuresis, of acid urine stained with phthalein, but the changes noted in the color of the tissues, cannot be ascribed to an unusually great elimination of dye: for the application of alkali to specimens from the control and experimental animal rendered them equally and deeply rose-purple.

Effects of Acid on the Coloration with Brom Cresol Purple.

An extensive series of observations were carried out with brom cresol purple. The blood and lymph did not alter notably in hue as acidosis developed, yet there were great changes in the surface color, the hue turning from the *dull violet-blue* of Ridgway to *grayish violet-blue*, or, if the animal was more deeply stained to begin with, from *deep soft blue-violet* to *grayish blue-violet*. The alterations were mainly the outcome, as further findings showed, of changes in the hue of the connective tissue.

Skin flaps dissected up in such wise as not to disturb the circulation markedly, appeared pale greenish blue in contrast with the intense purply blue of the control, when viewed under oil by transmitted light. The ears were purply green, not a frank purple as in the uninjected animal; and when segments were cut and rapidly compressed between mica slides to drive out fluid, the one appeared brilliant green and the other a frank purple. The extruded fluid, protected by oil, was in both cases purply blue. No better evidence of the independence of the tissue color could have been desired. Valentine knife sections of the ear of the experimental animal showed a blue-green, or even yellow-green, connective tissue, as compared with the deep blue of the control, and a light green, or greenish blue, cartilage, that of the control being a deep purply blue. The tendons, purply blue in the latter animal, were a paler blue or else green in the rat given acid, and a like contrast was to be observed in the wall of the femoral artery. These differences cannot be laid to a greater decolorization as result of the injection; for the application of alkali to pieces of the tissue showed that those from the injected animal contained as much and often more of the phthalein.

The color of the parenchymal organs appeared practically uninfluenced by the acid, save in the case of the kidney. The renal cortex, as viewed in the gross, had undergone an obvious change toward yellow, though the microscope showed much of the difference to be due to a more acid secretion, the fluid within the tubules being green, not purple as in the control. There was, in addition, some yellowing of the tubular epithelium. The pancreas, though, and the lymph nodes, never altered from the ordinary yellow-green staining, nor did the liver from its characteristic blue-green except in animals moribund from the acidosis, when it was noted to have changed very slightly toward yellow. The change may conceivably have been due to alterations in the reaction of the bile which was deeply stained (21).

It will be seen that the findings corroborated those with alkali in pointing to the existence of a group of tissues readily influenced in reaction by the introduction of acid or alkali into the blood stream, and of another group not susceptible to such change. And, as in the experiments with alkali, brom cresol purple gave information confirming and extending that obtained with phenol red.

The Changes Resulting from Injections of Lactic Acid.

Some subsidiary tests were made on the effects of lactic acid in isotonic solution (0.314 N) upon animals stained with phenol red. The injection fluid was tolerated in large amounts, 14.5 cc. failing to cause the death of a rat of 126 gm. into which it was run in the course of only a little over an hour; and unless a flow into the jugular was

continuously kept up not even a change in the surface color took place. By hurrying the injection a surface change from *jasper red* to a hue between *carnelian red* and *vinaceous-rufous* could be brought about, and under such circumstances alterations were to be noted in the color of the connective tissue, cartilage, and tendons, resembling those when hydrochloric acid had been used. But as soon as the injection was intermitted the surface color began to return toward the normal, as doubtless did the hue of the tissues mentioned, though observations were not made upon the point.

DISCUSSION.

The foregoing experiments make clear the fact that when acidosis or alkalosis is induced by the gradual injection of hydrochloric acid or of sodium carbonate into the blood stream changes take place in the reaction of some of the tissues. We have not sought to determine how great an amount of acid or alkali is required to cause these changes, nor have we made detailed observations upon the influence of the rate of injection. In most instances enough of the solution to cause death was eventually run in, but the examination was made while the animal was still in good condition save in the case of those tissues which the preliminary work had shown to be resistant to change. The solution could be allowed to enter rapidly at first, because of the buffering action of the blood, but later only drop by drop, so timed that each drop took some seconds or minutes to flow in. At first, too, because of the buffering action just mentioned, no change in the color of the tissues occurred. A few drops of the solution given late in the injection period often had more effect than did as many cc. earlier.

The question may be asked whether the changes were not the result of gross injury to the walls of the blood vessels with effusion of the acid or alkali. This possibility can be ruled out at once. The animal organism will tolerate but slight alterations in the reaction of the blood; and the color phenomena elicited by the injection of weak solutions (N/6.35) were the same as with strong ones (N/2 N/1). Further, the acid and alkali were of a kind to yield innocuous salts. It is true that even N/6.35 HCl acts to transform into acid hematin the hemoglobin coming directly into contact with it. But the damage

to the blood produced in this way under the circumstances of the experiments was not of itself sufficient to explain the issue of them. In a later paper some figures will be given on the anemia produced in rabbits by the intravenous injection of the maximum quantity of hydrochloric acid compatible with life. The blood loss is surprisingly slight.

How now are the changes in the relative reaction of the tissues to be accounted for? By changes in the ability of the blood to carry oxygen and carbon dioxide? This factor may have importance in the case of the animals given acid, owing to the effect of the latter to lessen the affinity of hemoglobin for oxygen, and thus to reduce the effective concentration of the gas. Asphyxia of the tissues is a well known cause of acid formation within them; and toward the end of some of our experiments the animals manifested air hunger. Acid lessens as well the ability of the blood to transport carbon dioxide; and the presence of this gas in the tissues of the stained, acidotic animal can be sufficiently shown by laying them open, when they rapidly become more alkaline, as evidenced by a change in hue. But this happens in the case of the similarly ventilated tissues of controls as well (22). When one alters the reaction of the blood in the direction of acidity one alters also the gradient responsible normally not alone for the passage of carbon dioxide from the tissues into the blood but for that of the acid products of metabolism generally. Here is yet another possible reason for the acidosis. This may have been due in no inconsiderable part to a damming back of substances which would normally escape to the circulation and be excreted, instead of to an extravascular penetration of hydrogen ions introduced with the injection fluid.

May one suppose that under the circumstances of blood alkalosis the gradient just referred to becomes so steep that the tissues are stripped, so to speak, of carbon dioxide and other acid products as soon as these are formed, with result that the local reaction becomes more alkaline than the normal? Such an occurrence is conceivable, since stained normal tissues ventilated by exposure to air soon become as alkaline as they ever appear to be after an injection of alkali.

The tissues which alter in reaction when alkali or acid is run into the organism,—connective tissue, cartilage, and tendon,—are those

characterized by the presence of matrix substance in large quantity, a matrix which stains deeply with the phthaleins. As pointed out in previous papers these tissues appear to be largely passive in behavior. They control their own conditions only where the influence of the separated cells is immediately effective; and when stained with phthaleins and immersed in weak buffer solutions they behave almost like so much indicator paper (23). Their metabolic rate is very low as compared with that of the parenchymatous organs. One may ask whether the intercellular substances which constitute so large a part of their bulk are alive at all in any proper sense of the word. It is easy to suppose that the changes in the reaction of these tissues are the direct result of an exchange of ions between them and the altered blood, without invoking other causes. But the precise interpretation of our findings cannot now be attempted for lack of data.

To give reasons for the failure of the parenchymatous organs to undergo changes in reaction is another, and more difficult matter. True, the cells of these organs may be thought of as making their own conditions (24), one of these being under ordinary circumstances,—or so it would appear,—a not inconsiderable acidity. But there is no evident increase in this local acidity in animals rendered acidotic, despite the great metabolic activity of the cells; and no diminution in it even when alkalosis is pushed to the extreme. It is possible, not to say certain, that the abilities of cells to maintain a normal state of affairs within their cytoplasm are often greater than those for survival of the body as a whole. Yet quite possibly too the phthalein of our experiments may in the parenchymatous organs have become so associated with some of the cell components that it no longer served as an indicator. All of the observations made in this laboratory prior to the ones here reported are against this explanation, however.

The changes toward acidosis or alkalosis sustained by the organism under the conditions of nature differ from those we have reported in that they are not ordinarily caused by a direct introduction into the blood of H or OH ions from without, though often referable to an indirect passage of them into the circulation by way of the gut. A large proportion of clinical instances of alkalosis or acidosis derive from some upset here or there amongst the organs. But except in the immediate region of the upset the net result must be the same in the

end in its effects upon the tissue reaction as if the disturbance had been a general one. That in pronounced cases an alteration takes place in the reaction of the matrix tissues can scarcely be doubted.

There is much on record to show that the introduction of acid into the body leads to a withdrawal of base from the tissues; and the quantity lost may well alter the composition of these latter. One wonders whether the matrix tissues, which are so readily permeable from the blood, may not serve as a second line of buffers, acting as interstitial tissues, tissues that fill up cracks, in a physiological as well as an anatomical sense. It would be in keeping with what is termed the body economy if a material like cartilage should prove useful in other ways than by keeping the ears at an angle from the head and the joints of the limbs well rounded.

Somewhere in the literature surely, there must be indirect observations upon the participation of the tissues in the buffering activities so beautifully illustrated by the blood; for the extent of the tissue participation can be come at, since the total blood value as a buffer is calculable and the changes incident to excretion can be controlled. The only observations we have been able to find, however, are those of Atzler and Lehmann (25) who perfused the isolated hind legs of dogs with acid and alkaline solutions over long periods of time and came to the conclusion that the greater the initial degree of acidity or alkalinity the greater was the change toward neutrality undergone by the perfusate.

Our observations by the indicator method upon the changes occurring in the tissues when acid or alkali has been run into the blood stream are not the first upon this theme. Henning (26) injected HCl or NaOH into mice and sought to determine the reaction of the organs after the method of Gräff (27) by dipping pea-sized pieces of them into drops of indicator solution placed on a glass slide. The animals had been decapitated, or had died as direct result of the injection; and the specimens stained after death contained the blood into which acid or alkali had been run. Under these conditions Henning noted that when a great deal of acid or alkali had been placed in circulation practically all of the tissue pieces except those from the brain appeared to contain H or OH ions in abnormal quantity.

Some of the rats of the present work withstood so well the introduction of N/6.35 HCl and the resulting marked changes in tissue reaction that we have been led to make observations upon the course of tissue acidosis as thus induced. The work constitutes an elaboration in certain directions of that here reported. It will be the subject of a succeeding report.

SUMMARY.

The introduction into the blood stream of dilute hydrochloric acid or sodium carbonate in quantities not too great to be compatible with life results in marked alterations in the color of certain of the matrix tissues stainable *in vivo* with phthalein indicators. Connective tissue in its various forms, and tendon and cartilage all become relatively more acid or alkaline than the normal. The hue of the kidney cortex also changes, as might be expected from its functions. The pancreas and lymph nodes, on the other hand, appear unaffected even when the injection is pushed to the extreme; and the slight to negligible alterations in the hue of the liver may be due to changes in the color of the associated secretion. The matrix tissues just mentioned behave as if unable to maintain a reaction of their own; whereas the elements of the parenchymal organs would seem to make their own conditions even when so much acid or alkali has been injected as will lead to death of the animal.

Injections of lactic acid are well tolerated and it is difficult to bring about alterations in the color of the phthalein-stained matrix tissues by means of them. Salt solution, and sugar in large amount cause no changes in color such as would indicate changes in reaction.

The results with the various phthalein indicators are in close accord, attesting that the information these give under vital conditions is reliable.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VIII. ON THE COURSE OF THE TISSUE ACIDOSIS SECONDARY TO BLOOD ACIDOSIS INDUCED WITH HYDROCHLORIC ACID.

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In a previous paper (1) the fact has been demonstrated that the reaction of certain tissues can be altered by injecting into the blood amounts of acid or alkali that are compatible with life. Rats vitally stained with a phthalein and receiving dilute hydrochloric acid survive despite marked changes in the color of the connective tissue, tendons, and cartilage. The abnormality in pH thus attested persists for a surprisingly long period. It has seemed possible, by repeating the experiments in larger animals, to learn something of the general relation existing between the reaction of the blood and tissues. Such experiments, upon rabbits, form the main subject of the present paper. But before they are taken up it will be well to give in detail a few observations on the effect upon rats of maximum quantities of hydrochloric acid.

Preliminary Experiments on Rats.

Experiment I.—At 9.20 a.m. a male rat of 123 gm., shaved over the body 24 hours previously, was given an intraperitoneal injection of 1.85 cc. of 4 per cent phenol red isotonic with the blood, but at pH 5.6. The animal was then etherized, a cannula connected with a jugular vein, the body immersed in a warm bath of oil, and, at 10.10 a.m., N/6.35 HCl was run into the circulation. The apparatus and general technic were those employed in a previous paper (2). The hue of the rat to begin with was deeper than *jasper red* (Ridgway) and no change was observed until after 3 cc. of the acid had been given, when the shaved surface appeared somewhat yellowish. When 5 cc. was in, at 10.37 a.m., it had become *coral red*. The breathing was exaggerated. By 11.05 a.m. 9 cc. had been injected and the animal was slightly lighter than *rufous*. Urine had been repeatedly voided in

large amount, at first red, then orange-red. At 11.10, with 9.5 cc. in, a movement of the animal during the course of the stormy respirations loosened the attachment of the cannula, and the vein had to be tied. The ether was discontinued and the rat taken from the bath. Within 10 minutes it was walking about. The surface tissues, now decolorizing, were still acidotic as shown by a *carrot red* color.

At 1.40 p.m. when the animal had completely decolorized a second injection of 1 cc. of phenol red was given. By 2.30 it was deeply colored, *carnelian red*. At 5.10 the injection was repeated. And now the hue was indicative of a return toward the normal, being between *jasper red* and *coral red*. During the afternoon the rat voided great quantities of intensely orange urine. Next morning, as well as 5 days later, it was in excellent condition. Healing of the neck incision was rapid.

Experiment II.—The rat weighed 124 gm. and received 1.85 cc. of the same phthalein solution used previously, soon after which it was etherized and placed in the oil bath. In the course of 1 hour and 40 minutes 14.8 cc. of N/6.35 HCl was run into a jugular vein. Toward the latter part of this period there was marked air hunger. 20 minutes after the injection,—and the ether,—had been terminated, with the animal already up and about, it was given 1.0 cc. more of the phthalein into the peritoneal cavity. Within a minute it died, as if from an embolus,—though none could be found in the pulmonary artery. A clot was present in the jugular below the tie. The color changes during the injection of acid had followed the general course of those noted in Experiment I.

Experiment III.—The test was repeated but in this instance the rat of 128 gm. was anesthetized with 1.1 cc. of 20 per cent urethane injected into the subcutaneous tissue of the back of the neck. 15 minutes later it received into the peritoneal cavity 1.9 cc. of 4 per cent phenol red at pH 7.4. When the injection of acid was begun 47 minutes had elapsed in all. During the course of the next 2 hours and 24 minutes 13.5 cc. was run in. The final hue was markedly acidotic, between *light ochraceous-salmon* and *light ochraceous-buff*. The animal died about an hour later. There was no respiratory distress at any time, in noteworthy contrast to the air hunger seen when such an experiment is done under ether.

Other similar observations were made which confirmed the findings described. When N/6.35 HCl is injected (3) the connective tissue and tendons change from yellow-pink and pinky yellow respectively to a clear orange-yellow. When the rats are stained with brom cresol purple instead of phenol red a change can be seen in the color of the cartilage, evidencing that it also has been rendered acidotic.

The Vital Staining of Rabbits with Phenol Red.

White rabbits can be brilliantly stained with phenol red by the injection into an ear vein of 5 cc. of a 4 per cent solution for every kilo of body weight. Within half an hour the body surface is an intense red, but soon afterwards decolorization begins, so rapidly is the dye eliminated. The animal behaves normally from first to last, and can be stained again and again without obvious injury. Most of the phthalein—as much as 96 per cent—appears in the urine within 24 hours of the injection.

The chest and abdomen of the rabbits were shaved a day or so beforehand in order to lessen the possibilities of error due to inflammation from abrasions. They were placed upon an electrically heated pad and were often covered lightly with a cloth to ensure further against an abnormal loss of body heat. The same apparatus was used to warm the injection fluid as in the case of rats but the tube leading to the vein passed directly from the water jacket to the cannula only a few centimeters away.

The color of the shaved body of the vitally stained rabbit tends to be slightly more alkaline than that of rats similarly treated. It varies with the individual from a very exceptional *jasper red* (Ridgway)¹ to *eugenia red* more commonly. The color changes can be appraised for purposes of record in terms of pH by a method already briefly described (4). An Autenrieth wedge filled with water is placed over the shaved and oiled surface of the abdomen or chest, care being taken not to exert pressure, and the color as thus viewed is compared with that noted when one or another of a series of similar wedges, filled with buffer solutions of known pH, colored with phenol red, is placed over the shaved surface of a control rabbit. By viewing through a slit the color of the various portions of the wedge placed on the control it is possible to obtain a match in color intensity as well as color character. Differences of much less than 0.1 pH can easily be read in this way. The wedges should be warmed to 35–38°C. The hue of the vitally stained but otherwise normal animal, as expressed on the pH scale, varies between pH 7.4 (*jasper red*) and pH 7.6+ (a hue slightly purpler than *eugenia red*). It will be seen that these individual variations have about the same magnitude as those which are known to occur in the normal blood. But needless to say our

¹ Ridgway's "Color standards and nomenclature" (Published by the Author, Washington, D. C., 1912) has been utilized in recording the colors, as in a previous paper of this series (Rous, P., *J. Exp. Med.*, 1926, xliv, 815). Wherever the hues provided by this book are mentioned in describing the findings italics will be employed.

use of pH figures to record the surface hue must not be taken to imply that the visible tissues have actually this pH, since there are many factors of complication. For one thing the surface is viewed by reflected light whereas readings of actual pH should be accomplished by transmitted light. The color of a fold of loose skin as seen by the latter method when it is pulled away from the body wall by gentle traction is of a different red from that observed on inspection of the same skin *in situ* by reflected light. It might be thought that the deeply stained blood would supply an important part of the surface color; but this is not the case. When all blood is washed out of the animal the surface is still an intense red (5); and whole thickness skin grafts stain heavily with the phthalein even during the period when they are completely avascular (6). One can safely conclude that the hue of the intact body surface in the living animal is referable to a distribution of the dye to the tissues; and it is upon this well justified assumption that our experiments are based. The precise whereabouts of the extravascular phthalein that renders the animal ruddy is another matter. So little of it is in the epidermis that this may be neglected; but much is in the tissue fluid,—as can be sufficiently shown by the change in color witnessed when the latter is forced out, as by compressing a skin fold between glass slides. A great deal is fixed upon the interstitial elements of the connective tissue (7). Whether the cells of this tissue contain any is doubtful.

To follow the changes in hue of the connective tissue, as distinct from those in the composite color of the body surface we have run a fine thread through the oiled skin here and there in regions where it was loose, as just above the groins; and at various periods of the experiment by pulling upon the thread have raised a fold and compressed it briefly between slides. The color of the cartilage could be followed in the ears when the circulation in them was good; but not infrequently it was so poor that the initial staining greatly lagged behind that of the body generally, as, too, did the changes consequent upon the administration of acid. In the rat the normal cartilage stains orange-yellow with phenol red, whereas in the rabbit it becomes pale pink. The more considerable alkalinity thus evidenced in the case of the latter animal is also encountered in the connective tissue which, in a compressed skin fold, appears a frank pink with only the faintest suggestion of yellow, not yellow-pink as in the rat.

By the technic described we were able to follow closely the extravascular changes in surface hue indicative of *relative* changes in pH; and, more roughly, the changes in color undergone by connective tissue and cartilage *in situ*. It remained to determine the reaction of the blood. This was accomplished by Hawkins' method (8).

The blood specimens were taken from the marginal ear vein directly into pipettes graduated to 1/100 cc., which had been ground to fit Luer needles. The latter were short, of 23 gauge, and they had been bent at a reentrant angle for con-

venience in piercing the vein in the direction of the ear tip. At the moment when this was to be done the operator made pressure upon the vein distal to the point of entry of the needle, releasing it at once thereafter so that the flow might be unimpeded. Since the vessels were distended by stroking or heat the specimens (0.25 cc.) must be thought of as consisting for the most part of arterial blood. They were procured in duplicate under oil and diluted in the usual way with salt solution. When the animal was heavily stained only enough phenol red had to be added to this solution to permit of the preliminary adjustment to pH 7.4. More of the phthalein proved necessary, of course, when the rabbit happened to be decolorizing. The protective layer of paraffin oil was replaced with melted paraffin (of low melting point) as soon as the blood and salt solution had been mixed. Readings were made in a colorimeter block at 38°C. The correspondence between the two tubes of any given specimen was, in our experience, absolute; and the whole procedure proved easy to carry out. The pipettes and needles required most careful cleansing else clotting was encountered. The same vein could be pierced again and again. This was essential to avoid using the ear into which phthalein had been injected.

The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly in pH, as determined with the potentiometer, from the calculated values. In charting the results corrections have been made for the differences, whereas in the protocols the figures as originally read off are recorded.

The Outlying Acidosis Attending Ether or Urethane Anesthesia.

In initial tests the rabbits were anesthetized with ether or urethane and they lay on the back throughout the injection period. Even under the best of conditions and in the absence of anesthesia the mere weight of the body proved sufficient to give rise to an acidosis of the surface tissues pressed upon, as was shown by their change in color from red to orange; and when the limbs were stretched out under tension the circulation on the surface of the abdomen was frequently interfered with and some acidosis developed there as well. But even in the absence of these sources of disturbance there was frequently a local acidosis to be observed in the anesthetized rabbits. On injection with phenol red after they had been unconscious for some time they colored up much less promptly than did normal animals; and large irregular regions over the body and in the ears remained untinted after the red staining had become pronounced everywhere else. When phthalein did at length penetrate into these regions their color had an orange cast. Evidently a local acidosis had developed in them, secondarily to an interference with the blood supply. As

is well known a blood acidosis develops during ether anesthesia. But independently of any such change, there was an extravascular acidosis, an "outlying acidosis" resembling that we have described in other connections (9).

Experiment IV.—Effects of urethane. 142 cc. of N/6.35 HCl was run into the jugular vein of a male white rabbit (No. 1) weighing 1625 gm., which had been given 3 cc. of 50 per cent urethane into the peritoneal cavity 2 hours beforehand. The injection took 1 hour and 40 minutes. When 50 cc. had been given 10 cc. of 4 per cent phenol red isotonic with 0.9 NaCl and at pH 5.6 was slowly introduced into an ear vein, without intermitting the flow of acid to the jugular. The animal stained only very gradually and irregularly *jasper red*, with patches coloring up later of *coral red* here and there. As time passed and the HCl injection proceeded the color altered more and more toward orange, and had become *rusous* in some places, *apricot orange* in others, by the time all the acid was in. The determinations on the blood showed a fall in pH from 7.4 prior to the giving of the acid to 7.0 immediately afterwards. Later the surface color became still yellower, between *zinc orange* and *ochraceous-salmon*, instead of altering toward red, and the animal died 1 hour and 40 minutes after the injection had been finished. From first to last there was no air hunger despite the acidosis. The surface hue at the end corresponded with pH 6.8.

In this instance not only was the staining irregular, with relatively acidotic areas here and there, but the complete absence of a compensatory increase in the breathing attested to a depressant effect of the urethane. To the absence of this respiratory compensation death was attributable, the animal becoming more and more acidotic after the injection was stopped, instead of tending to recover as happens after ether or in the absence of any general anesthesia. Cushny and Lieb have shown that the reaction of the respiratory center is altered in rabbits deeply under urethane (10).

Experiment V.—Effects of ether. The rabbit (No. 3) weighed 1600 gm. The first blood specimens, taken just prior to a light etherization, were at pH 7.4+. Now the jugular vein was cannulated, and 35 minutes after the blood had been taken the acid injection was begun. When 71 cc. of N/6.35 HCl had been run in the cannula was flushed with salt solution by means of a three-way stop-cock connecting with it, and 10 cc. of 4 per cent phenol red isotonic with 0.9 NaCl and at pH 7.4 was gradually injected. The animal colored only very gradually, *jasper red* here, *coral red* there, with a streak of orange-yellow along the midline from ensiform to symphysis. As more and more acid was injected the respirations became stormy and the general color changed to *apricot orange* variegated with

rufous. After 160 cc., given in the course of 2 hours, the jugular was tied and the ether, which for some time had been almost left off, was wholly discontinued. The *second blood specimens* were now taken, reading midway between pH 6.8 and 6.9. The *surface hue* on the other hand corresponded with 7.0— to 7.0+. The animal soon got to its feet and shortly thereafter the staining lost its patchy character. During the rest of the day it appeared in good condition save for the exaggerated respirations. Several blood specimens were taken. These showed only a slight return toward a normal reaction whereas there was a more marked extravascular return as shown by the surface color. The changes find record in the chart (Chart 1).

The animal was given food and left for the night. Next morning it was reported to be decolorized and in good condition, having eaten well. Unfortunately it was placed in a cage with other animals to be brought to the laboratory, and, as it moved about amongst them, it suddenly collapsed and died, making violent respiratory efforts. Apparently its slight exertions had been sufficient to overturn the *status quo*. At autopsy no anatomical cause for death was found.

In this experiment (Chart 1), which was duplicated by another, the injection of an enormous amount of the acid solution was withstood by the animal. There was the stormy respiration usual in acidosis, a feature which had been lacking with urethane, and after the injection the animal tended to recover as had not been the case when the latter was used. The staining with phenol red, done at a time when considerable acid had been run in, disclosed a poor circulatory condition of the tissues, as shown by a tardy distribution of the dye and the presence of relatively acidotic areas over chest and abdomen. The rapid change to an even staining after the animal had come out of the anesthetic was a significant feature as was also the return of the surface color toward the normal during a period when the blood reaction remained almost unchanged. As the later work showed, this return was referable to the disappearance, as the circulation became better, of a generalized "outlying acidosis" which had been present in addition to the extravascular acidosis induced by the HCl. In none of the later experiments, in which a local anesthesia took the place of a general one, was any pronounced color patching noted like that with ether or urethane; in none did the hue become as orange,—despite the fact that the injection of acid was pushed to the extreme; and in none did the extravascular reaction return more rapidly to the normal than that of the blood.

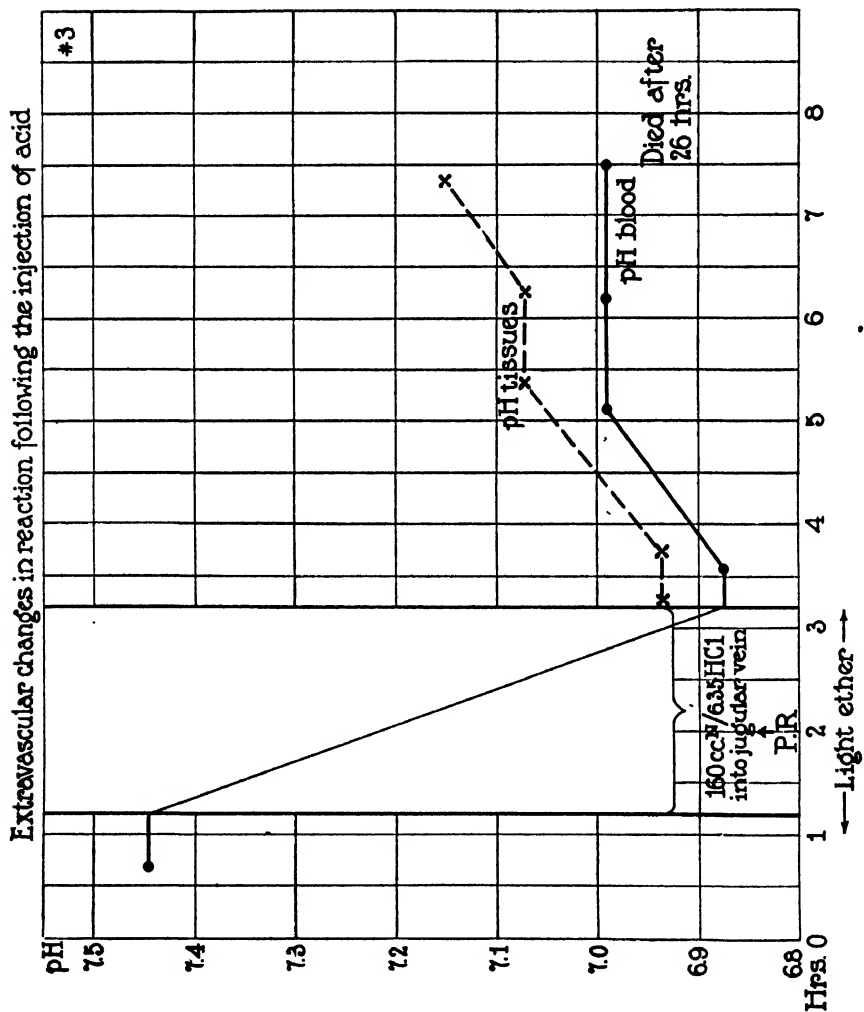


CHART 1.

For the purpose of the foregoing observations the animals were stained with phenol red only after the injection of acid had proceeded for some time; since otherwise the staining did not last long enough to cover the entire later period of study. The plan was now adopted of giving smaller amounts of the indicator with repetitions of the dose at more frequent intervals. Tests directed to the point had shown that the intravenous injection of 10 cc. of 4 per cent phenol red, brought to pH 7.4, exerted not the slightest effect on the reaction of the blood. To expose the jugular vein novocaine was employed, without precautions for sterility. After the injection was finished the neck incision was sutured and the animal placed by itself in a metabolism cage where it could be carefully watched. Abstracts of two protocols will be given.

Experiment VI.—(See Chart 2.) Male white rabbit (No. 6) of 1690 gm. 9.30 a.m.—*First blood specimens taken.* 9.37—First injection of phenol red. 10.05—Rabbit coloring rapidly and evenly. 10.22.—*Surface hue* is old rose; by wedge method pH 7.5. 10.30—*Second blood specimens taken.* 11.29—*Injection of N/6.35 HCl begun* at rate of 5 cc. every 3 minutes. 12.06 p.m.—65 cc. in. Animal completely decolorized. 12.35—102 cc. in. Pause for injection of phenol red, 10 cc., by way of the jugular cannula, between 12.36 and 12.39 p.m. Injection of acid now renewed at a slower rate. 12.56—115 cc. of acid has been injected. The animal is everywhere *jasper red*. 2.29—The general color is *coral red* but with caroty areas here and there. *Injection terminated and animal removed from pad.* It has received 170 cc. of acid in all. 2.50—*Third blood specimens taken.* Animal O.K. 3.00—*Surface hue* between *coral red* and *carneian red*; by wedge method pH 7.2. 4.33—*Fourth blood specimens taken.* 4.39-40—Injection of phenol red, 5 cc., into an ear vein. 5.13—*Surface hue* by wedge method pH 7.3—. 5.18—*Fifth blood specimens taken.* 5.41—*Surface hue* by wedge method just about pH 7.3 (artificial light).

During the latter part of the period of injection the respiratory amplitude was greatly increased. The animal remained quiet throughout, however. Afterwards it voided large quantities of urine colored orange-red with phthalein. During the night it escaped from the metabolism cage, and some urine may have been lost. Next morning the respirations were still as exaggerated as on the previous evening, and the animal was weak and apathetic.

Second Day, 9.55 a.m.—*Sixth blood specimen taken.* 10.08—Injection of phenol red, 5 cc. into an ear vein. 10.20—The rabbit has colored but poorly, yet there is no evident outlying acidosis. 10.39—The coloration is diffuse; *surface hue* by wedge method, pH 7.3+. 11.18—*Seventh blood specimen taken.* 11.33—*Surface hue* by wedge method, pH 7.3. Respirations still exaggerated. 12.07—There is

a sudden commotion in the cage, and the rabbit previously quiet, is found in opisthotonos. It died at 12.10.

The autopsy showed atelectasis of the upper lobes of both lungs, and in the right psoas muscle a contracted blood clot apparently about 24 hours old with a volume of about 10 cc.

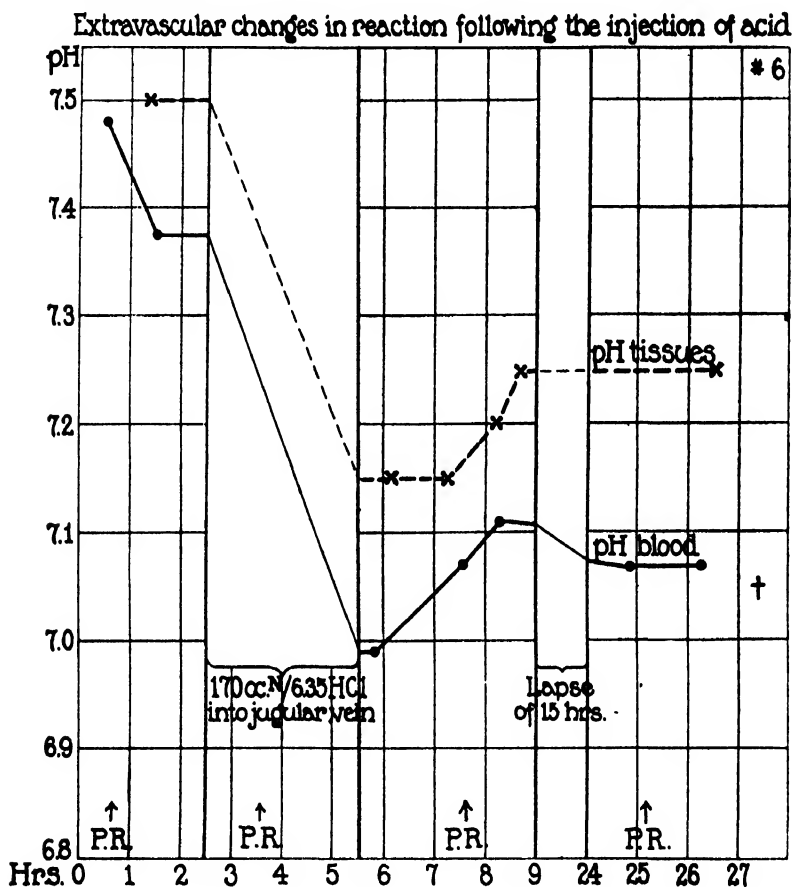


CHART 2.

The bladder had been emptied by catheterization at the beginning of the experiment. From then until death 184 cc. of urine was secreted, an amount somewhat larger than the total of acid solution injected. The rabbit had free access to water during the night and to food but appeared not to have partaken of either. The amount of circulating hemoglobin fell from 78 per cent to 58 per cent (Newcomer), and the red cells from 6,590,000 to 3,490,000 per c.mm. in the first 24 hours. A part of the blood change is attributable to the hemorrhage into the muscle.

In this experiment the maximum quantity of acid was given that could be borne. The reaction of the blood was altered from pH 7.4 to pH 7.0, and during the next 24 hours it scarcely changed (Chart 2). The surface color, recorded in terms of pH, gave readings that were regularly further on the alkaline side than those of the blood; but, as has already been emphasized, these readings cannot under the conditions be taken as expressive of the actual pH prevailing. They have merely a relative value as indicative of the extravascular condition. The change in this would appear to have been not quite so great as in the blood, the apparent pH falling from about pH 7.5 to pH 7.2. The later, slight recovery in the blood was duplicated, however, by an extravascular recovery of equal magnitude.

The death of the animal cannot be attributed directly to the acidosis because of the lesions found at autopsy. The question comes up, indeed, as to whether the persistence of the acidosis may not have been due to these latter. That it was not, the following experiment shows.

Experiment VII.—Male rabbit (No. 7) of 1650 gm. Jugular bared under local anesthesia with novocaine. 9.42—*First blood specimens taken.* 9.50½–52—Injection into an ear vein of 4½ cc. phenol red. 10.22—*Surface hue* between *old rose* and *eugenia red*; by wedge method, pH 7.5. 10.53—*Second blood specimens taken.* 11.02—*Surface hue* by wedge method pH 7.5. 11.46—*Injection of N/6.35 HCl begun.* 12.36 p.m.—59 cc. has been injected. 1.13—Injection of 5 cc. phenol red by way of the cannula in the jugular. The animal colored evenly and quickly, *jasper red*. 1.46 p.m.—132 cc. of acid in. *Surface hue* between *light jasper red* and *coral red*. 2.02 p.m.—142 cc. of acid in. Rabbit is restless; breathing greatly exaggerated. 2.19—Animal nearly decolorized. 2.24 p.m.—149 cc. in. 2.24–26½—10 cc. of phenol red injected into jugular. A rapid and deep staining developed. 3.01 p.m.—158½ cc. of acid has been run in; *injection terminated* and incision sewed up. 3.10—*Surface hue* between *coral red* and *jasper red*. 3.11—Rabbit removed from board. It is alert but stands weakly, and has a notable air hunger. 3.26—In excellent condition, though still weak and with air hunger. *Surface hue* now slightly darker than *carrot red*. 3.43—*Surface hue* by wedge method between pH 7.1 and pH 7.2. 3.52—*Third blood specimens taken.* 4.00—*Surface hue* again between pH 7.1 and pH 7.2. 4.51—*Surface hue* just above pH 7.1. 4.57—*Fourth blood specimens taken.* 5.03—*Surface hue* between pH 7.1 and 7.2. 5.20—Decolorizing. Left overnight, with water but no food. Eager to eat cabbage shown it.

Second Day. 9.35 a.m.—Animal in excellent condition except for weakness of fore leg on side of jugular injection. Breathing still exaggerated. 9.44—*Fifth*

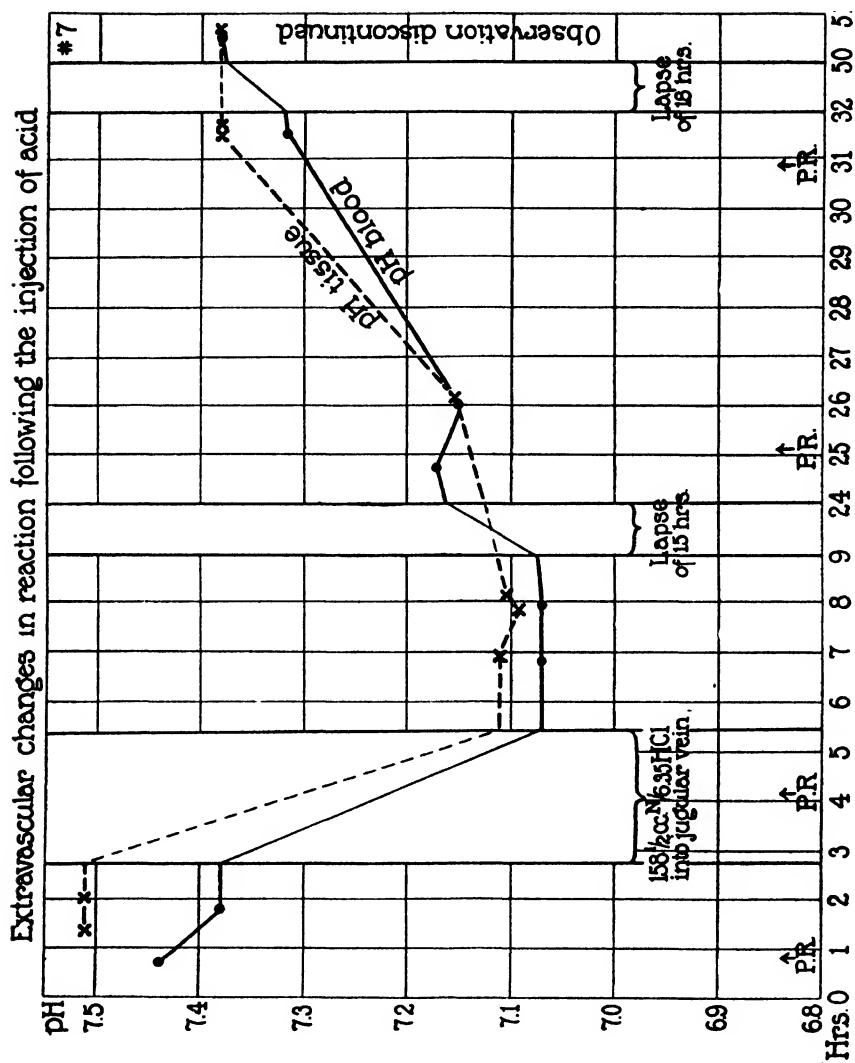


CHART 3.

blood specimens taken. 10.04-06—7 cc. of phenol red injected into an ear vein. The staining that followed was rapid, even, and deep; *surface hue* by wedge method pH 7.3. 10.41—*Surface hue*, by wedge, just below pH 7.3. 11.00—*Sixth blood specimens taken.* 11.04—*Surface hue* by wedge, pH 7.2. 11.09—Color is between *light jasper red* and *light coral red*, slightly darker than either. 11.32—Given cabbage which it eagerly attacks. 2.15—Has eaten largely; condition excellent though right fore leg is still dragged. 3.53—Injected with 7 cc. of phenol red. 4.14—General color *jasper red*. 4.32—*Surface hue* by wedge, pH 7.4. 4.35—*Seventh blood specimens taken.* Still some air hunger on exertion. 4.42—*Surface hue* pH 7.4. 6.13—Left overnight with food. General condition as before.

Third Day. 10.24 a.m.—Has again eaten largely. Injected with 7 cc. phenol red. 10.35—Given more cabbage; eating. 11.07—*Surface hue* by wedge pH 7.4, and again pH 7.4 at 11.24. 11.28—*Eighth blood specimens taken.* *Surface hue* by wedge pH 7.4. Decolorizing; color is *old rose*. 12.27—Not yet fully decolorized; observations terminated.

Fourth Day. Weight 1500 gm.

Fifth Day. The tissues of the neck are indurated about the old wound. Animal killed with chloroform. The general condition of the organs is excellent save in the case of the kidneys, the renal cortex appearing somewhat swollen and paler than normal (parenchymatous degeneration?).

The bladder had been emptied by catheterization prior to the injection of acid, and during the 19 hours thereafter 223 cc. of urine heavily colored with phthalein was voided. During this period no food had been given.

The hemoglobin dropped from 75 per cent prior to the experiment to 58 per cent on the next morning, and the number of red cells from 5,460,000 to 4,430,000. In the afternoon the hemoglobin percentage was 65 and the number of red cells 4,750,000. On the 2nd day the figures were the same. For the determinations and those of Experiment VI we are indebted to Dr. Charles A. Doan.

In this experiment (Chart 3) the alteration in the surface color, as recorded in terms of pH, was slightly greater than that found in the blood, whereas in the preceding one it had been slightly less. The correspondence between the later extravascular changes, indicated by the surface hue, and the intravascular ones was exceedingly close. The observer following the alterations in the blood did not know of those in the surface hue. The variations in pH were almost identically the same in both, and the same very gradual recovery took place until food (cabbage) was given, when the acidosis rapidly lessened, almost disappearing within a few hours. How long recovery might have taken had no materials to combat the acidosis been supplied by mouth is problematic.

The amount of urine voided by the animal during the first 19 hours after the acid injection (223 cc.) considerably exceeded the fluid bulk introduced in this way (158½ cc.).

The diminution in the amount of blood in circulation attested by the lessening in hemoglobin and in the number of circulating red cells is to be attributed in no small part to the repeated bleedings incident to the observations on the blood reaction. The remaining loss was doubtless due to an action of the hydrochloric acid solution on such red cells as came into direct contact with it during the injection period.

Significance of the Extravascular Changes.

The alterations in the surface color that took place during the foregoing experiments might be attributed wholly to changes in the reaction of the stained extravascular fluid did not incidental observations show that the hue of the tissues themselves was altered. We have already described the method whereby it is possible to determine the hue of connective tissue *in situ*. Oiled folds of skin of the vitally stained rabbit when viewed between slides by transmitted light are ordinarily pink even when the fluid has been driven out of them by pressure upon the slides. After the injection of acid they appeared orange-pink until pressure was made when they were seen to be of a clear orange-yellow, far more acid, judging from the color, than the body surface as viewed by reflected light. The conjunctiva likewise became orange, and the sclera palely so. The cartilage in the least vascular portions of the ears of vitally stained rabbits is pink ordinarily. But after the acid injection it was a clear orange-yellow. In proportion as the general acidosis was recovered from, so too did all of the tissues mentioned alter from orange to pink. On the inside of the ear where the cartilage is covered with merely a film of adherent skin its color can be readily followed by reflected light; and the observation was made that at times when the alteration in the hue of the body surface generally had progressed no further than *coral red* the cartilage had become a clear orange. The finding would suggest that despite the relative alkalinity of normal rabbit cartilage as compared with that of the rat or mouse, an alkalinity finding expression in a pink staining with phenol red,

this tissue is still not so alkaline as the connective tissue, which in turn is less alkaline than the blood (11). The pH of normal rabbit blood is almost identical with that of the rat (12).

DISCUSSION.

The findings show that when changes in the reaction of rabbit blood are induced with hydrochloric acid extravascular changes of approximately the same magnitude occur. No dissections were made to inspect the interior organs; but from previous experiments on rats it seems certain that conditions of blood acidosis or alkalosis leading to a change in the color of connective tissue and cartilage will also lead to one in the color of the tendons, whereas the hue of liver, pancreas, and lymph nodes will be unaffected even when the blood change is so great as to lead to death (13). There is every reason to suppose that the same holds true in the case of rabbits, and that by following the visible alterations in the connective tissue and cartilage one can obtain an index to much of what would be disclosed by dissection under oil.

The failure of the extravascular reaction to alter more than does that of the blood would at first sight seem surprising. For, with a lessened gradient between tissues and blood, one might suppose that there would be some heaping up of the acid products of metabolic activity. Perhaps such a heaping up and not a passage of H ions from the blood is actually one of the causes of the extravascular acidosis. Whenever the circulation is interfered with, as during ether and urethane anesthesia, a heaping up of acid metabolites certainly does occur, with result in an "outlying acidosis" independent of that due to the injected acid. One is reminded in this connection of Yandell Henderson's statement that most patients and animals under general anesthesia for more than an hour are in the first stage of shock (14). Our experiments prove that under the conditions of such anesthesia an abnormal local reaction develops within some of the tissues which may well prove detrimental to the organism as a whole.

The demonstration that a widespread extravascular acidosis occurs when there is a marked blood acidosis, and that it is tolerated for

long periods has a bearing upon the problem of shock. For many years now the relationship between the functional state of the small blood vessels and the reaction of their milieu has been debated; and many have supposed that a vascular dilatation is caused by acidosis and that such a dilatation may be responsible for shock through the changes in the distribution of blood that it entails. The British Commission for the investigation of surgical shock could not produce it by injecting acid into the circulation (15); but they were unable to bring proof that in altering the condition of the blood they also altered the reaction outside of the lumen of the vessels. That extravascular changes go hand in hand with those taking place in the blood has been demonstrated in the course of the present work. Yet not the least evidence of shock was to be seen in our animals though the blood reaction was diminished to the limit compatible with life and the extravascular reaction of large regions was proportionately affected. Though the connective tissue was markedly acidotic, as shown by its color during the periods of staining, not the least vascular dilatation could be observed in it through the shaved and oiled skin. In the intervals of decolorization between the injections of phenol red there was good opportunity to make observations on the latter point.

The quantities of fluid voided by our animals during the many hours that the tissue acidosis endured regularly exceeded the very large amounts introduced with the acid. These animals had access to water but were given no food, and they certainly did not drink largely. There was not the least sign of a fluid retention in the acidotic tissues. In a previous paper (16) one of us has demonstrated that local acidosis and edema sometimes occur together but without any relationship of cause and effect. The present findings afford another example of the phenomenon.

SUMMARY.

The changes in blood reaction caused by the injection into a vein of a weak solution of hydrochloric acid are accompanied by extravascular changes of similar magnitude within the subcutaneous tissue. Under the conditions of prolonged general anesthesia with

ether or urethane the circulation to this tissue is so interfered with that an "outlying acidosis" may develop in addition to the acidosis immediately consequent on the blood state. Even under the best of circumstances the extravascular acidosis induced with hydrochloric acid affects not merely the tissue fluid but the reaction of the tissue itself.

Rabbits in which a widespread extravascular acidosis has been produced, together with a blood acidosis as severe as is compatible with life, remain in good condition during the relatively long period over which this state of affairs persists. There is at no time any sign of capillary dilatation, though the vessels are bathed in relatively acid fluid, and none of shock. No edema develops in the acidotic tissues, and the animals void large amounts of urine. The tissue acidosis lessens *pari passu* with that of the blood.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

IX. ON THE TISSUE REACTION AS INFLUENCED BY INHALATIONS OF CO₂ AND BY OVERBREATHING.

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In preceding papers from this laboratory the influence upon the tissue reaction of injections into the circulation of weak solutions of acid and alkali has been recorded, and the relation existing between the blood changes and those in the tissues during the course of an acidosis induced in this way has been described (1). We have now sought to determine the effect upon the tissues of breathing high concentrations of carbon dioxide, and of the overventilation that results from forced respiration.

The Inhalation Apparatus.

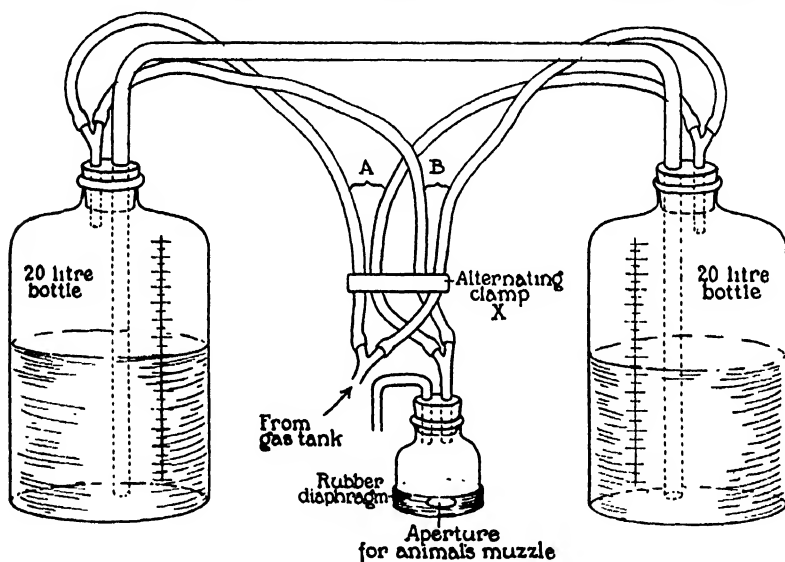
White rabbits were used. The general technic of vital staining with phenol red and of appraising the reaction of the blood and the changes in the color of the body surface have already been detailed (2). The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly in pH, as determined with the potentiometer, from the calculated values. In charting the results corrections have been made for the differences, whereas in the protocols the figures as originally read off are recorded.

A special apparatus was necessary, for the purpose of the inhalations of carbon dioxide. It is portrayed in Text-fig. 1.

The gas from the storage cylinder escaped into one or the other of two 20 litre bottles graduated in half litres and about half full of water weakly acidulated with hydrochloric acid. The pressure under which the gas passed into the one bottle was sufficient to force water over into the other through the large tube connecting the two, and the only way of escape for the carbon dioxide already in this other was through a tube which led to the inhalation mask fixed upon the animal's muzzle. By means of a special snap clamp (*X*) acting, now on the tubes *A*, and again on *B*, the direction of flow from the storage cylinder could be alternated to

the bottles at will without interruption of it. An observer with a stop-watch controlled the speed at which gas passed to the inhalation mask. One litre per minute was the ordinary rate.

The mask was made out of a short broad bottle from which the bottom had been cut. Across the opening thus provided a diaphragm of rubber was stretched with a hole in the center for the rabbit's muzzle. There was a continuous current of gas through the bottle, and the space in it was small, to prevent rebreathing. The tube through which the gas escaped was long in order that there might be no sucking back of air into the mask on inspiration. To ensure a snug fit of the rubber diaphragm all hair was removed from the animal's muzzle with a sodium sulfide solution. The mask was held in place by a strip of adhesive tape passing



TEXT-FIG. 1.

around the back of the head, and there was never any leakage between it and the skin. Care was taken not to interfere with the circulation to the ears.

Five experiments were performed with a mixture containing 21.08 per cent of CO_2 , and approximately 22.09 per cent O, and 56.83 per cent N. A single set of observations were made with 37 per cent CO_2 , 22 per cent O and 41 per cent N. For the analyses of the gas mixtures, as supplied in pressure cylinders, we are indebted to the kindness of Dr. C. A. L. Binger.

In four of the five experiments just referred to, urethane was employed as anesthetic. The blood specimens for the pH determinations were procured from a

femoral vein. In the case of the remaining animal, which received no general anesthetic the small incision necessary to bare the vein was made after infiltration of the tissues with novocaine. To aspirate blood Luer needles bent at a reentrant angle were used, as in the previous work. It was essential that the circulation of the leg furnishing the blood should not be interfered with, so no thong or other tie was placed about it. The vein could be punctured again and again in the groin without any important bleeding or thrombosis, if the needle was thrust slantingly through its sheath and pressure applied for a few moments after the aspiration.

In several of the experiments a minimum dose of urethane was employed and a far better respiratory response to the acidosis was obtained than in the animals more heavily anesthetized by its means and given hydrochloric acid (3).

The Acidosis on Breathing 21 Per Cent of CO₂.

The protocol of *Experiment 1* need not be given since the facts are better illustrated by the later work.

Experiment 2.—Male rabbit No. 2, of 1650 gm., given 3.3 cc. of 50 per cent urethane subcutaneously into the neck at 9.20 a.m., and 1 cc. more at 10.35, into the peritoneal cavity. 11.00—10 cc. of 4 per cent phenol red isotonic with 0.9 per cent NaCl and at pH 7.4 was injected into an ear vein. 11.50—Rabbit for some time on the warm pad. *Surface hue* slightly less purple than *eugenia red* (Ridgway); by wedge method between pH 7.5 and 7.6. Skin folds compressed between slides are pink by transmitted light with faintest yellow admixture; ear cartilage pale pink. 11.54—*First blood specimens taken*. 12.00—*Surface hue* between pH 7.5 and 7.6.

12.01—*Mask adjusted and gas run in*. The respirations at once mounted from 40 to 88 per minute, much exaggerated; but almost as rapidly they lessened in frequency again.

12.05—*The surface hue* generally has become yellower, between *jasper red* and *light jasper red*. No indication of spotting. 12.07—Respirations 56 per minute, regular. 12.10—*Surface hue* yellower than *jasper red*. Skin folds orange-yellow. 12.12—*Surface hue coral red*. 12.41—*Surface hue light coral red*. Skin folds pronouncedly orange-yellow; ear cartilage light yellow. 12.48—*Surface hue* by wedge method, pH 7.2. 12.51—*Second blood specimens taken*. 12.55—*Surface hue* by wedge, pH 7.2. 12.56—*Surface hue* still *light coral red*. During this long inhalation period the respirations have been much exaggerated but never stormy. Their rate has lessened from 56 to 48 per minute.

12.57—*Mask removed*. The respirations at once became much less ample, 52 per minute. Practically at once, too, the *surface hue* became pinker, the change being very definite by 12.59.

1.01—Animal stirred, as it had not during the inhalations. 1.03—Skin fold much less yellow. 1.08—*Surface hue* now approaches *old rose*. 1.12—*Surface hue* by wedge, pH 7.4. 1.14—Ear cartilage more pink than yellow now, while skin fold is as pink as before the inhalations. 1.19—*Surface hue* between *jasper pink* and *old rose*, pH 7.4 by wedge. 1.26—*Third blood specimens taken*. 1.30—*Surface hue* by wedge pH 7.4—; between *light jasper red* and *jasper pink*. Observations discontinued. The respirations varied between 52 and 40 after the mask was off; they were quiet.

Experiment 3.—Performed on afternoon of same day as Experiment 2 and upon the same animal, which was still unconscious owing to the urethane. 2.52—Given 8 cc. of phenol red into the same ear vein as before. 3.17—*Surface hue* slightly purpler and deeper than *jasper red*, by wedge method pH 7.5+. 3.19—*First blood specimens taken*. Ear cartilage is light pink; skin folds, compressed, are pink, with faintest yellow tinge. 3.23—*Surface hue* by wedge, pH 7.5+.

3.28—*Mask adjusted and inhalations begun*. The respirations, which, had averaged 48 per minute, quickened greatly at once but soon slowed again, to 62, much exaggerated.

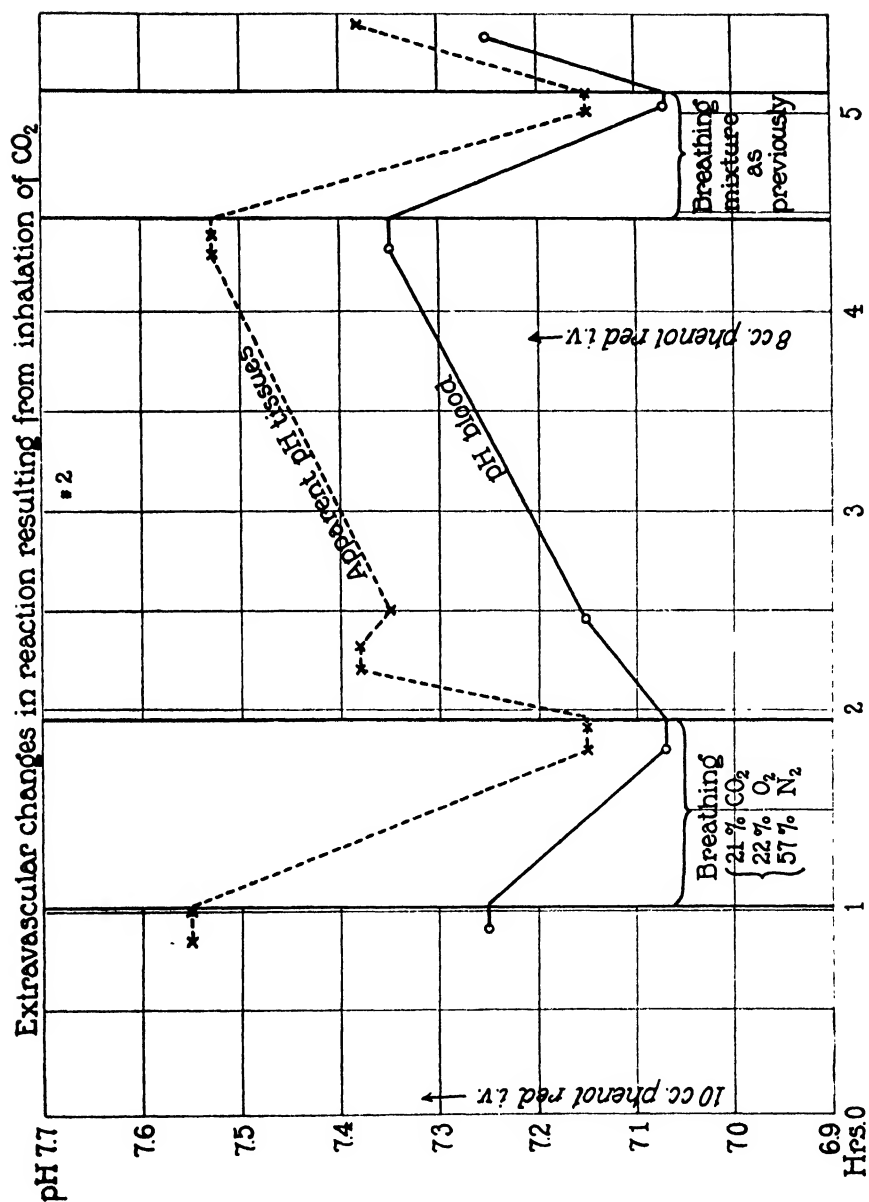
3.32—*Surface hue* yellower. 3.33—Skin folds much yellower. Some surface patching of color. 3.35—The general color is *jasper red*, with some *coral red* patching over chest and abdomen. The patches are irregular, only a few centimeters across. They have the general character of those seen in outlying acidosis. 3.43—The *coral red* patches have become confluent, everywhere driving out the rosy hue. A skin fold is bright orange-yellow but the color of the ear cartilage has not changed. 3.53—*Surface hue* pH 7.2+ by wedge method. There is a definite yellowing of the ear cartilage. The circulation in the ear is excellent. 4.00—*Surface hue* still that of pH 7.2+. 4.02—*Second blood specimens taken*. 4.07—*Surface hue* between *coral red* and *light coral red*; by wedge method pH 7.2+. Ear cartilage yellow. During the inhalations the respirations gradually slowed from 62 to 44 per minute, markedly exaggerated always.

4.08—*Mask removed*. Immediately the breathing became stormy and quick for a few moments, but then quieted and slowed to a rate of 48 per minute and gradually, by 4.30, to 32 per minute. The color of the animal at once began to change back to the normal, being definitely more ruddy by 4.11. The change was generalized.

4.13—*Surface hue* between *jasper red* and *light jasper red*; skin fold pink, with but a slight admixture of yellow; ear cartilage still yellowish. 4.19—*Surface hue* between *light jasper red* and *old rose*. 4.25—*Third blood specimens taken*. 4.28—*Surface hue* by wedge, pH 7.4. 4.30—Animal stirring slightly.

Observations discontinued. 50 cc. of warm water given by stomach tube. Next morning the rabbit was in excellent condition, eating.

The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly, on test with the potentiometer, from the calculated pH values. Corrections have been made for the differences in charting the results, whereas in the protocols the figures originally read off are given.



As the protocols and the chart show (Chart 1) there was in these experiments not only a great alteration in the reaction of the blood but one in the extravascular reaction as well, when the animal inhaled a gas mixture containing 21 per cent CO_2 and the normal quantity of oxygen. The changes in the surface color indicative of an increasing acidity began practically at once, and, progressing rapidly, reached a maximum some little time before the inhalations were discontinued. When this had been done an immediate return toward the normal took place, at first rapid, then more gradual. In Experiment 3 there was, as the general acidosis developed, a patching which resembled that of outlying acidosis.

Experiment 4.—Male rabbit No. 4 of 1700 gm. given 4.5 cc. of 50 per cent urethane into the peritoneal cavity at 9.20 a.m. and 10 cc. of 4 per cent phenol red into an ear vein at 10.04. 10.25—Staining is even and deep; animal on warm pad, deeply anesthetized. 10.48—*Surface hue* not quite so purple as *eugenia red*; by wedge method at pH 7.5. 10.54—*First blood specimens taken*. 10.57—*Surface hue* still at pH 7.5. A skin fold is pink with slightest tinge of yellow; ear cartilage pink.

11.08—*Inhalations begun*. Immediately the respirations increased in rate and amplitude.

11.12—*Surface hue* is yellower. 11.14—*Surface hue* now *jasper red* save for a longitudinal streak about 4 cm. wide extending from ensiform nearly to symphysis, which is still purply red. 11.15—Skin fold more yellow. Respirations still markedly exaggerated but of nearly the same rate as before the inhalations were begun, 48 as compared with a previous 44 to 46 per minute. The rate altered practically not at all from now until the mask was removed. 11.23—Dubious yellowing of ear cartilage. Good circulation in the ear. 11.29—*Surface hue* between *jasper red* and *coral red*. 11.33—Skin folds are much more yellow; and so too is the ear cartilage. 11.40—*Surface hue* generally is between *coral red* and *light coral red*. The median streak of differing color has disappeared. 11.48—Circulation to ear is excellent yet cartilage is orange-yellow, as also are the skin folds. 11.52—*Surface hue* by wedge method pH 7.2+. *Second blood specimens taken*. 11.59—*Surface hue* at pH 7.2; yellower than *coral red*. Color of skin folds and cartilage orange-yellow.

12.00—*Mask removed*. Respirations temporarily increased in amplitude and frequency, being 60 to the minute at 12.01 but soon becoming quiet and slowing to 40. Practically no change took place in the *surface hue* for a long time, however. At 12.37 it was still between *coral red* and *light coral red*; and at 12.40, by wedge method, at pH 7.2.

12.46—*Third blood specimens taken*. 12.49—*Surface hue* by wedge pH 7.2. 2.00 p.m.—Rabbit still deeply unconscious, an even *light jasper red*. Skin surface

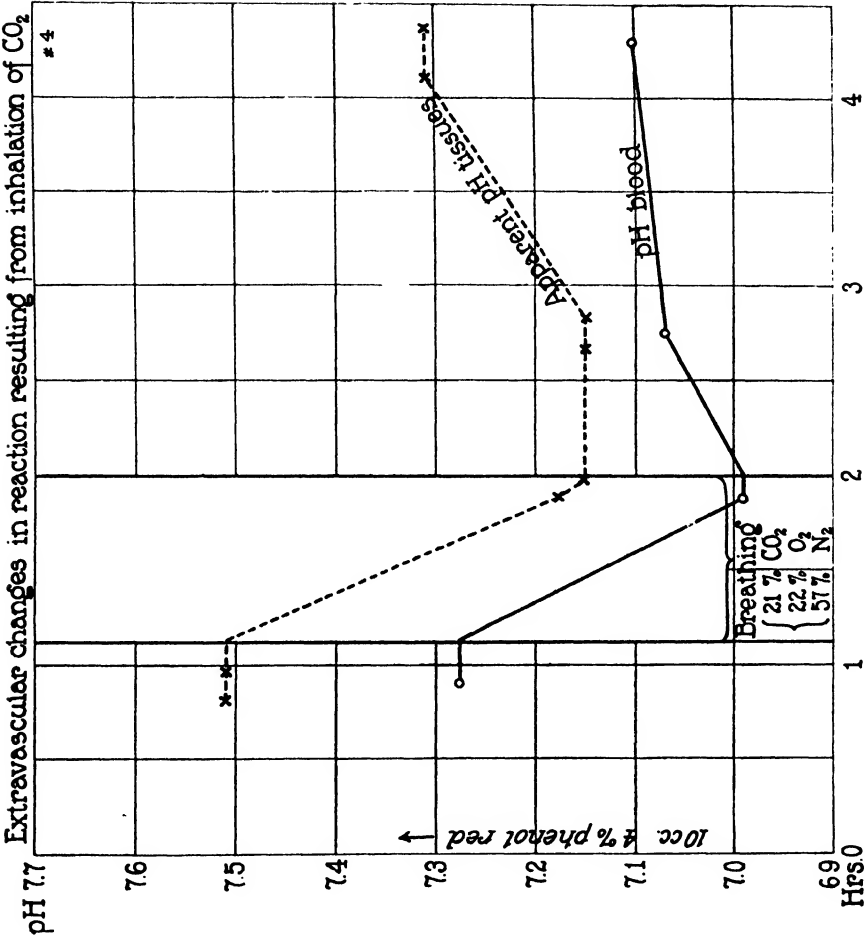


CHART 2.

is warm. 2.07—*Surface hue* between pH 7.3 and pH 7.4. Skin folds still much yellower than normal. Ear cartilage has decolorized too far for appraisal of the hue. 2.18—*Fourth blood specimens taken*. 2.24—*Surface hue* by wedge between pH 7.3 and pH 7.4. 2.25—*Surface hue* between *light jasper red* and *old rose*. Skin fold still is yellower than "normal." Animal has not stirred at all. 5.00—Color still a medium light pink. Animal deeply unconscious. Observations discontinued. 50 cc. of warm water was given by gavage and next day the rabbit was in excellent condition.

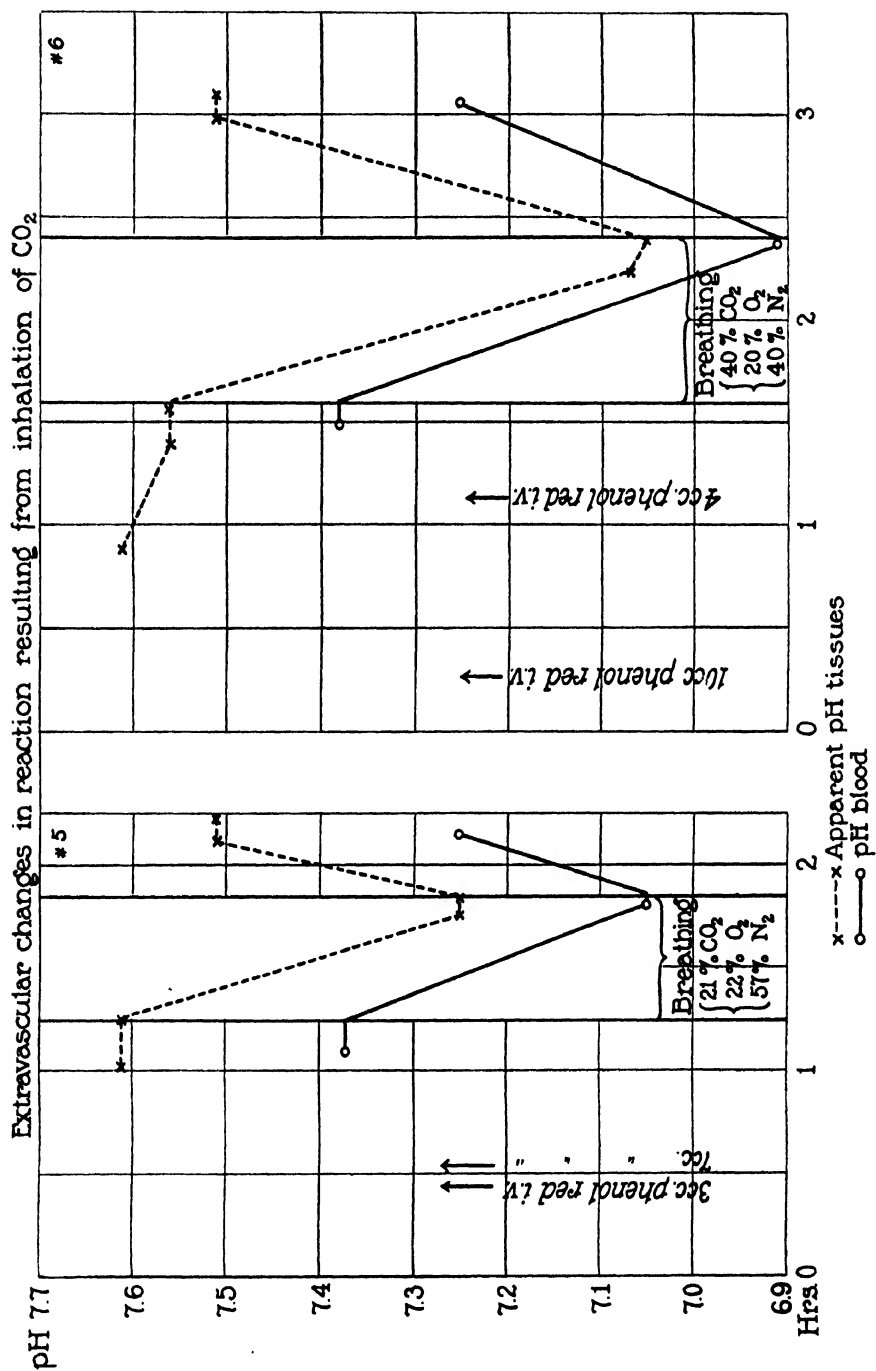
The injection of urethane into the peritoneal cavity resulted in a deeper anesthesia than when the same dose was given subcutaneously (Experiments 2 and 3). The rabbit never moved during the observations, and the staining persisted for an unusually long period. But more important was the persistence of acidosis after the mask had been taken off (Chart 2). Comment has already been made (4) on the action of urethane to lessen or prevent the compensatory respiratory response that is usually called forth by an acidosis. As result of this occurrence the acid products of metabolism tend to heap up within the organism to a greater extent than would follow merely from the introduction of acid from without. In the present case the extravascular acidosis indicated by the surface hue was not unusually marked; and hence one can scarcely attribute its persistence to an unusual accumulation of acid metabolites. Doubtless the depression of the respiratory activities persisted after the mask had been taken off.

As a check upon the findings observations were now made in the absence of a general anesthetic.

Experiment 5.—Male rabbit No. 5 of 1500 gm. 10.25–30 a.m.—10 cc. of phenol red given into an ear vein. The animal at once began to color up rapidly and evenly. It was placed on the warm pad and a femoral vein bared with the aid of novocaine. 11.02—*Surface hue* slightly less purple than *eugenia red*; by wedge method at pH 7.6. 11.05—*First blood specimens taken*. The animal is quiet. 11.10—Skin flaps are pink, with the slightest yellow admixture. The cartilage of the ear is deep pink. 11.14—*Surface hue* by wedge still at pH 7.6.

11.15—*Inhalations begun*. The breathing at once became rapid and stormy but the animal did not struggle then or later. The respiratory rate rose abruptly from 80 to 110 but gradually fell again, reaching 78 at the end of the inhalation period. Throughout it the breathing was greatly exaggerated.

11.17—*Surface hue* rapidly losing its purple quality. Some linear razor marks heretofore not visible are now rendered suddenly prominent by an orange border



to either side of them. 11.20—*Surface hue* nearing *jasper red*. 11.23—There is a pronounced spotting over the abdomen, of many orange-red patches about 1 cm. in diameter, scattered on a more ruddy background. The veins are nowhere turgid. 11.25—The patching has disappeared and the acidotic hue is confluent save for a broad streak from ensiform to pubis which is still purply red. The streak corresponds with the region where a poorly vascularized aponeurosis underlies the skin. The skin flaps are a marked orange-yellow and so too is the ear cartilage. The circulation in the ear continues excellent. 11.29—*Surface hue* between *coral red* and *light coral red*. The inside of the ear, where a bloodless cartilage is but thinly overlain with skin, is no longer pink by reflected light but yellow. The scleral conjunctiva has turned ruddy orange. There is an excellent conjunctival reflex and the animal appears conscious. 11.38—*Surface hue* slightly lighter than *coral red*; the median streak of more purply red has almost completely merged in the general hue. Skin flaps are clear orange-yellow; ear cartilage orange-yellow. 11.44—*Surface hue* at pH 7.3 by wedge method. 11.49—*Second blood specimens taken*. 11.50—*Surface hue* at pH 7.3.

11.50—*Mask removed*. Immediately the respirations became even more ample than before and the animal appeared excited. The superficial veins over the abdomen and chest became greatly engorged even in the finer ramifications.

11.52—*Surface hue* redder and conjunctiva has become pink again; but skin flaps are still orange-yellow. 11.54—Animal has now quieted and so too have its respirations. 11.57—*Surface hue* between *eugenia red* and *old rose*. The venous congestion continues. 11.59—The skin flaps have returned to the usual pink with faintest admixture of yellow; the ear cartilage is much pinker. 12.06—*Surface hue* as at 11.57; by wedge method at pH 7.5. 12.08—*Third blood specimens taken*. 12.15—The animal has struggled at intervals but remains quiet during repair of the groin incision. The veins over chest and abdomen are still notably congested. 12.17—Circulation in the ears is greatly cut down, and the cartilage is still somewhat yellow both by transmitted and reflected light. 2.40—Animal in excellent condition, very light pink. 3.05—Animal decolorized except for the ears which show a light pink cartilage. The superficial veins are still engorged. Observations discontinued. The animal was let up, when it behaved normally.

The results of this experiment (Chart 3) corresponded in general with those of the preceding ones. The tissue acidosis did not become so great, however, and recovery from it was more prompt, though not quite complete when the observations were terminated.

The Acidosis on Breathing 37 Per Cent of CO₂.

During the latter part of the inhalation periods of the foregoing experiments the surface hue changed but little. It was evident that the

limits had been reached of the extravascular acidosis that could be induced with 21 per cent CO_2 . In order to obtain more marked changes resort was had to a mixture containing 37 per cent of this gas.

Experiment 6.—Male rabbit No. 6 of 1500 gm. No general anesthetic. At 10.16 a.m. 10 cc. of phenol red was given into an ear vein and a little later the animal was placed on the warm pad and a femoral vein bared with the aid of novocaine locally. 10.52—*Surface hue* slightly less purple than *eugenia red*; by wedge method at pH 7.6. 11.08—Beginning to decolorize, so 4 cc. more of phenol red given into same vein. 11.23—*Surface hue* slightly yellower than at 10.52; by wedge method between pH 7.5 and pH 7.6. 11.30—*First blood specimens taken*. 11.33—*Surface hue* slightly above pH 7.5. Skin flaps are pink with a trace of yellow, and ear cartilage also. The circulation in the ear is good.

11.35—*Mask on and inhalations begun*. The animal held its breath for about half a minute, then struggled, soon became quiet, and began to breathe regularly and very deeply. During the inhalation period the respiratory rate gradually lessened from 68 to 42 per minute.

11.38—*Surface hue* rapidly becoming yellower, nearing *jasper red* except along midline of abdomen, where as yet there is little change. 11.40—Ear cartilage much yellower. 11.41—Skin flaps already ruddy orange. 11.42—*Surface hue*, generally, *jasper red*. 11.51—Ear cartilage orange-yellow. 11.52—*Surface hue coral red*. 11.53—Animal appears to be completely unconscious; slight rhythmic movements of legs; good conjunctival reflex. 12.00—*Surface hue* about midway between *coral red* and *carnelian red*. 12.01—Skin flaps more orange. 12.05—Bloodless ear cartilage yellow by reflected light. 12.09—*Surface hue carnelian red*. 12.14—*Surface hue* by wedge method at pH 7.1. 12.22—*Second blood specimens taken*. 12.23—*Surface hue* slightly yellower than *carnelian red*; by wedge method pH 7.1—.

After 12.00 n. the accessory muscles of the neck were called into play during the respirations. By 12.18 these were labored and less extensive. For fear that a gradual failure of ventilation might ensue and complicate the findings the mask was removed at 12.24. At that time there were 42 breaths to the minute. The color of the animal was still changing toward orange. Immediately that the mask was taken off the respirations became much shallower and the rate rose to 82 per minute. The rabbit, previously unconscious to all appearance, raised its head at 12.26 and struggled at 12.27. The ruddy color was now rapidly coming back.

12.28—*Surface hue* approaching *coral red*. 132 respirations per minute, shallow. 12.33—Rabbit quiet; 100 respirations per minute. *Surface hue* here *jasper red*, there *coral red*. 12.36—Conscious but quiet. 12.43—*Surface hue* only slightly yellower than *old rose*. 12.47—Ear cartilage still definitely yellower than ordinary. 12.48—Skin flaps have the normal pink hue. 12.58—*Surface hue* is *old rose*; by wedge method at pH 7.5. 1.03—*Third blood specimens taken*. 1.05—*Surface hue* by wedge, pH 7.5. Experiment discontinued. During its course the rabbit had

lost somewhat less than 5 cc. of blood by a slow escape from the femoral vein. When seen next morning it was in excellent condition.

The effect of breathing an atmosphere containing 37 per cent CO_2 with about the ordinary quantity of oxygen was to render the animal unconscious, and to induce a progressive acidosis (Chart 3). Toward the end of the inhalation period there were signs of respiratory failure; and complications from this source would doubtless have occurred had the mask not been taken off. The extravascular acidosis induced was more considerable than with 21 per cent CO_2 and apparently its limits were not reached. The acidosis of the blood became as pronounced as is ordinarily compatible with life, according to other investigators. Recovery was prompt but not quite complete during the brief period of observation.

Effects of Overventilation.

As a corollary the influence upon the tissue reaction of overbreathing was studied. It was already known that flaps of living and well vascularized connective tissue become more alkaline when exposed to air (5). So too does the peritoneal lining. With overbreathing sufficient to reduce considerably the carbon dioxide tension of the blood one would expect some change in the extravascular reaction. Such a change was obtained, a definite but not a marked one as would follow from the fact that the induced blood alkalosis was but slight. Six experiments were performed, upon rabbits.

Under general anesthesia, brought about with urethane in all save one instance, the animals were tracheotomized and a limb of a T-tube passed down nearly to the bronchial bifurcation and tied in place. In the exceptional case ether was used during the tracheotomy and the lips of the incision were swabbed with novocaine prior to discontinuance of the general anesthesia, at the time when the experiment proper was begun. The tube that formed the staff of the T was connected with the house suction in some instances and in others left open, while the free limb was connected with a motor-run mechanism devised by Dr. F. L. Gates, whereby air, separately warmed and moistened, was blown continuously or rhythmically into the lungs. The chest of some of the urethanized animals was opened by a bloodless incision down the middle of the sternum and a screw retractor inserted to expose the lungs. The blood specimens were taken from a femoral vein, and the surface hue appraised by the wedge method as usual.

Blowing air continuously for half an hour through a catheter with its opening down almost as far as the bifurcation of the bronchi was without effect on the surface hue of the animal stained with phenol red; and when the stream of air was cut off no apnea ensued. Filling and emptying the lungs by the alternate blowing in of air, and suction upon the tracheotomy tube with the house vacuum resulted in well defined changes but great care had to be taken to control the pressure relations, else pulmonary hemorrhage ensued. The best results were obtained in animals with relatively flexible thoracic walls which permitted of a large expansion and deflation of the lungs. It was found that when the chest was opened the animal developed the signs of a marked outlying acidosis despite the existence of an overventilation as proven by the apnea that ensued when the artificial respiration was stopped. In our opinion this acidosis resulted from a peripheral vascular constriction, secondary to and compensatory for, an interference with the circulation which was in turn traceable to an embarrassment of the heart. This last organ,—no longer provided with its usual orientation and supports,—collapsed upon itself at each deflation of the lungs.

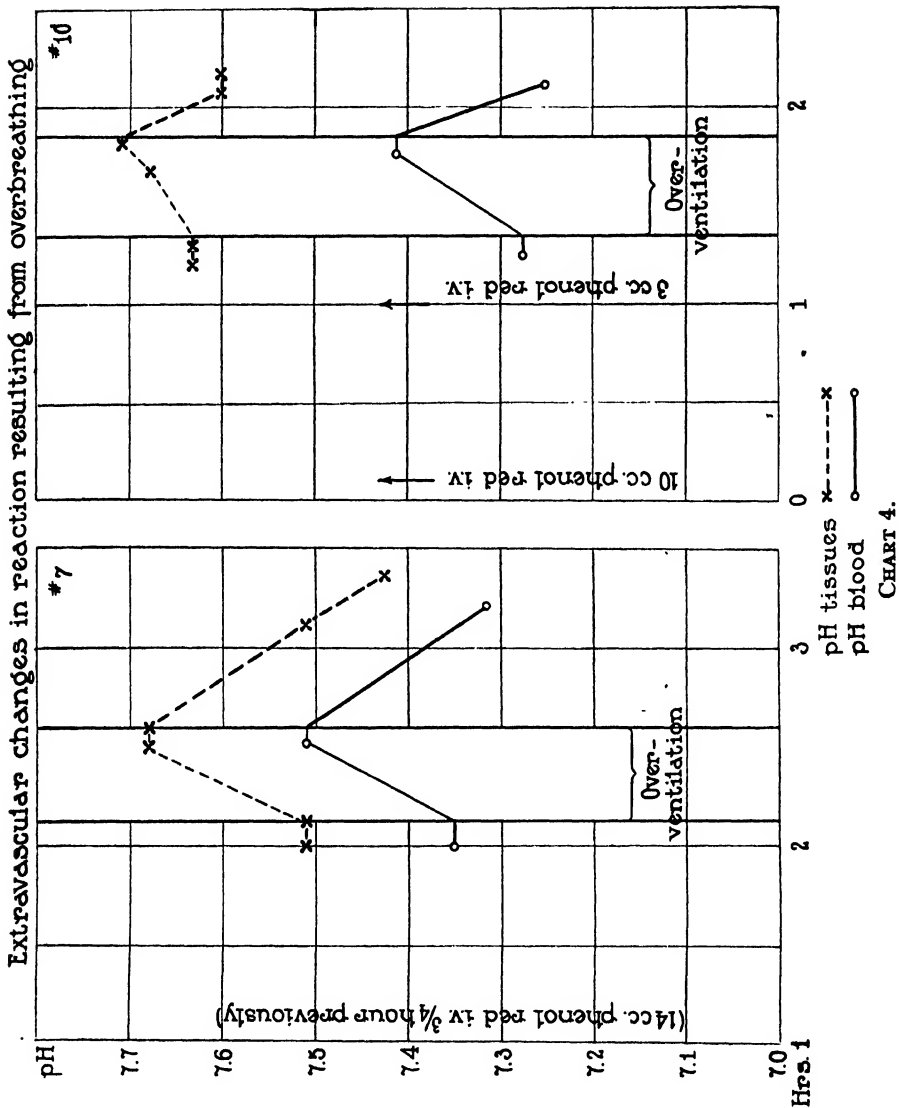
The alterations in the reaction of the blood and tissues were never great; and the surface hue was more difficult to appraise in terms of pH than was the case in the acidosis experiments, because the colors were well toward the alkaline side of the range of phenol red. The colors could not always be recorded precisely in terms of Ridgway's nomenclature because the values associated with alkalosis differed somewhat from his scales. Two protocols will be given.

Experiment 7.—(a) Male rabbit No. 7 of 2075 gm. given 4 cc. of 50 per cent urethane into the peritoneal cavity at 12.02 and 10 cc. of phenol red into an ear vein at 1.08. A few whiffs of ether were administered during the tracheotomy, which had been accomplished by 1.27 p.m. 1.37—*Surface hue* between *eugenia red* and *jasper red* but deeper than either; by wedge method pH 7.5—. Skin flaps pink, with faintest yellow admixture.

1.40.—*Artificial respiration by pressure and suction begun.* The suction was sufficient to cause marked depression at the costochondral junctions and the normal respiratory rhythm was easily overcome. 1.46—On intermitting briefly the artificial respiration an apnea ensued lasting 45 seconds. 2.00—*Surface hue* deeper than *eugenia red*; by wedge pH 7.6+. 2.01—Skin flaps deep purple red, no yellow.

2.23—*Discontinued artificial respirations.* *Surface hue* at this time just above pH 7.6 by wedge. Apnea for 70 seconds; then resumption of breathing at 44 respirations per minute. During the suction and compression the rate had varied between 28 and 32. 2.27—*Surface hue* returning toward *eugenia red*. 2.39—*Surface hue* between *eugenia red* and *jasper red*. 2.55—*Surface hue* less purple and slightly lighter than *eugenia red*. Skin flaps once again show a tinge of yellow in the prevailing pink.

(b) 3.00 p.m. of same day—*Surface hue* by wedge pH 7.5. 3.01—*First blood specimens taken.* 3.05—*Surface hue* by wedge pH 7.5—. Skin flaps colored as before. Respirations 42 per minute.



Artificial respirations begun at 32 per minute. 3.08—Animal decolorizing. *Surface hue* only slightly darker than *old rose*. 3.17—*Surface hue* now much darker and purpler than *eugenia red*. 3.31—Decolorization proceeds. *Surface hue* lighter than *eugenia red*; by wedge midway between pH 7.6 and 7.7. 3.32—*Second blood specimens taken*. 3.34—*Surface hue* by wedge between pH 7.6 and 7.7.

3.35—*Artificial respiration stopped*. Apnea of 50 seconds. 3.37—Respirations 146 per minute, *surface hue* much less purple. 3.59—Respirations have gradually become slower, now 54 per minute. *Surface hue old rose*, by wedge pH 7.5. 4.13—*Third blood specimens taken*. 4.20—*Surface hue alizarin pink*, by wedge pH 7.4+.

At 4.26 the animal was killed with ether and autopsied. It showed two localized pulmonary hemorrhages, 0.5 and 1.5 cm. in diameter respectively, one in each lower lobe.

Experiment 8.—Male rabbit No. 10 of 1600 gm. Given 1.3 cc. of urethane into tissues of back of neck at 12.30; 1 cc. more into peritoneal cavity at 2.00; and a few whiffs of ether during the tracheotomy, which was accomplished by 2.30. Between 2.21 and 2.25 10 cc. of phenol red was injected into an ear vein. The vital staining was even and deep, but owing to time lost in some accessory observations it was necessary to give 3 cc. more of the phthalein, at 3.30, to compensate for the decolorization that was taking place. 3.43—*Surface hue* slightly lighter than *eugenia red*, at pH 7.6 by wedge. 26 respirations per minute. 3.47—*First blood specimens taken*. 3.48—*Surface hue* again, pH 7.6.

3.50—*Artificial respirations begun* at rate of 28 per minute by alternating pressure and suction. 3.58—The blood in the superficial veins appears much purpler, so that these are more clearly seen. (The like observation was made in other experiments not reported here in detail.) 4.11—*Surface hue* by wedge between pH 7.6 and pH 7.7. 4.16—*Second blood specimens taken*. 4.19—*Surface hue* purpler and lighter than *eugenia red*, by wedge pH 7.7—.

Artificial respirations stopped. Apnea for only 35 seconds. Thereafter the respirations were at first rapid, 140 per minute, and then gradually slowed, to 82 per minute. 4.24—*Surface hue* lighter and only faintly purpler than *eugenia red*. 4.34—*Surface hue* slightly lighter and faintly purpler than *old rose*; by wedge method pH 7.6—. 4.38—*Third blood specimens taken*. 4.40—*Surface hue* by wedge pH 7.6—. Respirations 86 per minute. Observations discontinued. The rabbit was chloroformed for examination, on the same day. No lesions were found other than a liver coccidiosis.

Changes in the color of the connective tissue and cartilage could not at any time be made out.

While the alterations in reaction produced in these experiments (Chart 4) were not great they were sufficient for the purpose in hand, namely to determine whether an extravascular alkalosis is linked with

the blood alkalosis induced by overbreathing. Such is the case, the alterations in the blood reaction being closely paralleled by similar extravascular ones, as attested by the surface hue. In one of the two experiments the purple hue of skin flaps compressed between slides and viewed by transmitted light indicated the development of a more alkaline reaction of the connective tissue. No such change was to be seen in the ear cartilage.

DISCUSSION.

The experiments show that under the circumstances of acidosis due to the inhalation of carbon dioxide, and of alkalosis consequent on a blowing off of the gas, changes occur in the extravascular reaction which closely parallel those in the blood. Without doubt the extravascular changes largely involve the interstitial fluids, but during the acidosis concomitant alterations take place as well in the connective tissue and cartilage, the matrix tissues so readily rendered acid by the intravenous injection of acid solutions. During the alkalosis we noted connective tissue changes only, but this is not surprising since it was slight in degree and of brief duration. In our experience alterations in the reaction of cartilage follow rather tardily upon those of the connective tissue (*vide* Experiment 3); and this is especially true of the cartilage in the ear of the rabbit, which is almost avascular in the regions best suited to inspection. No observations were made upon the deep-lying organs; but one would expect from the results in rats given hydrochloric acid (6) that under the circumstances of acidosis due to carbonic acid the tendons would also become acid. Whether the reaction would also change in the liver, pancreas, and lymph nodes, parenchymal organs unaffected apparently by even the most extreme hydrochloric acid acidosis, is an interesting question. Quite possibly this would happen. For carbon dioxide manifests abilities to penetrate living tissue far beyond those of other acids (7).

An extravascular acidosis began to develop practically at once on the inhalation of the carbon dioxide, whereas it did not appear until a large quantity of hydrochloric acid had been run into the blood. This was to have been expected since the introduction of hydrochloric acid is compensated for in large measure by the elimination of carbon dioxide through the lungs, a possibility excluded when the gas is

being taken into the body by the pulmonary route. But over and above this difference one may attribute the prompt development of the extravascular CO_2 acidosis to the known ability of the gas to penetrate tissues rapidly (7). Evidence of such penetration was clearly to be seen in the course of our experiments. Where the circulation was unusually good, as for example about the almost imperceptible abrasions due to shaving (Experiment 5), the acidosis developed soonest; and where the vascularization was scanty, as along the line between ensiform and symphysis (Experiments 4 and 5) there it was seen last. The patching with color sometimes witnessed during the course of the inhalations (Experiments 3 and 5), and resembling that of outlying acidosis, was in reality consequent on a diametrically opposite state of affairs, those parts becoming most rapidly acid, and in consequence standing out in orange-red against a red background, which were most accessible to the acidotic blood, not least so.

The continuance of a carbon dioxide acidosis would doubtless lead to some accumulation of acid metabolites here and there in the body; but during the brief period of our experiments little evidence of such an event was obtained. The reaction of the blood and tissues swiftly changed for the better when the inhalations were discontinued, in significant contrast to the persistence of acidosis when hydrochloric acid had been administered. True, the reaction did not quite return to the normal even under the best of conditions (Experiments 5 and 6), and a compensated acidosis may well have been present and have endured for some time. To determine the actual case was no part of our work which had for sole aim the immediate influence upon the tissue reaction of alterations in the CO_2 content of the blood.

The normal reaction of the blood, as obtained from the femoral vein in the present experiments, varied from about pH 7.25 to slightly less than pH 7.4, whereas that from an ear vein, as noted in our preceding work (8), ranged from just below pH 7.4 to nearly pH 7.5. This very considerable discrepancy between the reaction of the two sets of specimens read in precisely the same way and by the same observer was a consistent finding. The fact may be recalled in this connection that the blood had come from very different regions. Since our object was to follow the relative, not the actual, variations

in intravascular and extravascular reaction the problem thus raised will not be discussed.

Hawkins has shown that urethane anesthesia results in an alkalosis (9); yet there can be no doubt that under uncomplicated conditions it tends to prolong the acidosis resulting from the inhalation of carbon dioxide (Experiment 4, Chart 2), as also that induced by hydrochloric acid. There is evidence that this comes about through a change in the respiratory center (10).

SUMMARY.

Breathing an atmosphere that contains the normal amount of oxygen but a large excess of carbon dioxide results in a tissue acidosis as well as one of the blood. The extravascular changes in reaction take place with far greater speed than when acidosis is induced with hydrochloric acid, and they do not persist as in the case of this latter but swiftly disappear when the animal breathes ordinary air once again. The changes parallel closely in magnitude and time those occurring in the blood. The same matrix tissues are rendered acidotic as when hydrochloric acid is administered.

The blood alkalosis that results from a blowing off of carbon dioxide is accompanied by an extravascular alkalosis. Under the circumstances of our experiments the connective tissue became more alkaline than ordinary but no change was noted in the cartilage, a fact to be explained by the slight degree of the alkalosis and its brief duration.

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STUDIES IN BLOOD COAGULATION.

I. CERTAIN CHARACTERISTICS OF COAGULATION AND THEIR MEASUREMENT.

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PLATE 1.

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In the study of a malignant transplanted tumor of the rabbit (1, 2) it has been observed that animals pursuing a rapidly fatal course of the disease frequently show as a terminal event evidences of an hemorrhagic state, with extravasation of blood into muscles, connective tissue spaces and serous cavities. Blood tests demonstrate prolonged clotting rate and imperfect clot retraction. In earlier stages, also, of this florid and of milder forms of the tumor are found changes in blood coagulability, less extensive and consistently related to various phases of the tumor growth. The findings are to be reported in Part II of this communication, it being necessary first to consider in detail the characteristics of blood coagulation that were investigated and the means adopted for their evaluation.

In estimating blood coagulability particular attention has to be paid to the circumstances of the technique employed, since they of themselves may influence the readings very considerably. This has been emphasized abundantly by others, referring to the markedly accelerating effect of "tissue juices" upon coagulation (3), the acceleration produced by various foreign substances, metal, glass, etc., coming in contact with the specimen during the test (4), the retardation in clotting rate resulting from periodic or continuous disturbance of the blood necessary in making observations (5), or from exposure to the air, also, the important part played by the temperature at which coagulation takes place (6). While the effects of extraneous factors of this sort may be maintained constant in a given technique and the readings obtained be of significance relative to each other, the

result may be to remove the scale of values so far from the basic rate of coagulation as to obliterate certain abnormalities that occur. For example, cases of hemophilia have been described (7-9) in which the clotting time of blood taken directly from the circulation was greatly extended, while specimens that had touched the tissues in being secured coagulated at normal rates. These were extreme instances but they illustrate the importance of obtaining as nearly as possible a basic clotting rate value; and for the present work where but slight changes in blood coagulability are frequently presented such factors have had to be eliminated from the tests, a condition not satisfied by any of the procedures known to us. Effort has been made, accordingly, to develop methods with conditions of technique nearly neutral in all regards to coagulation. Indicators were sought for to serve as bases of measurement, phenomena occurring distinctively and spontaneously in blood coagulating at rest, rather than the arbitrary type of standard usually employed, such as certain degrees of firmness, adhesiveness, ductility or plasticity of the clot.

The problem at hand required consideration of two phases of the blood coagulative process, *i.e.*, clot formation and clot retraction. These will be dealt with separately; first, the characteristics of each will be discussed, and then methods of measurement described.

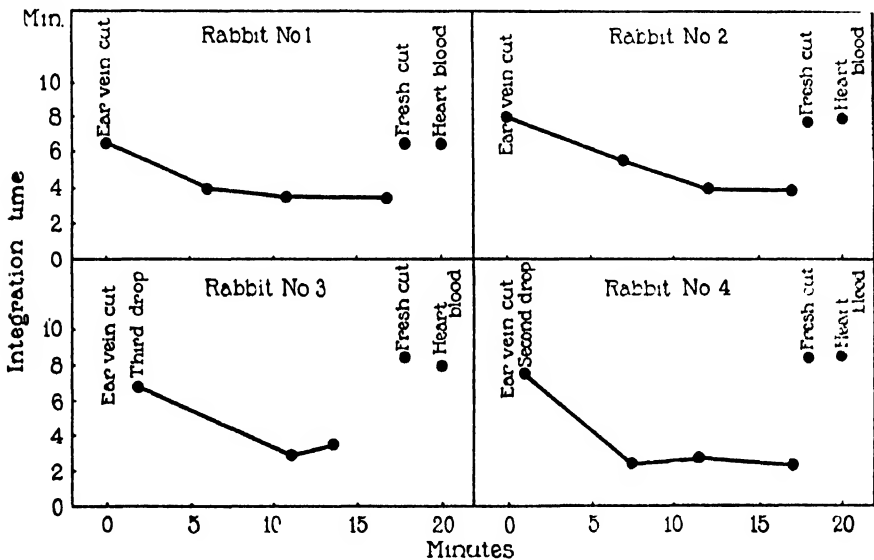
I. Clot Formation.

Experimental Basis.—A drop of freshly shed blood deposited under clear, bland oil and resting upon paraffin remains intact as a slightly flattened sphere and presents an exquisitely smooth and sensitive surface (Fig. 1, No. 1). Observed at room temperature, the sphere of blood remains without change of appearance for about 15 minutes, when, almost imperceptibly at first, retraction of clot begins as a minute dimple or wrinkle at one point or another (Fig. 1, No. 2), or as a warping of the whole specimen. Soon the distortions multiply and extend, and in the space of a minute or so the drop loses completely its symmetry and finish (Fig. 1, No. 3). Further retraction follows and bubbles of serum are extruded (Fig. 1, Nos. 4 and 5).

It is here evident that clot retraction commences in a specimen of blood only after a considerable period has elapsed since shedding, during which time the clot is being formed. Apparently, the fibrin

coagulum must undergo a certain amount of development or maturation before it becomes capable of retraction, and, once this condition has been attained, retraction proceeds rapidly. The duration of this period will be referred to as clot formation time, and, with the above preparation, it will be measured with the onset of retraction as end-point.

In the rabbit it is not practicable for repeated tests to take specimens of blood directly from the circulation by puncture of large vessels, and section of the ear vein must be employed.

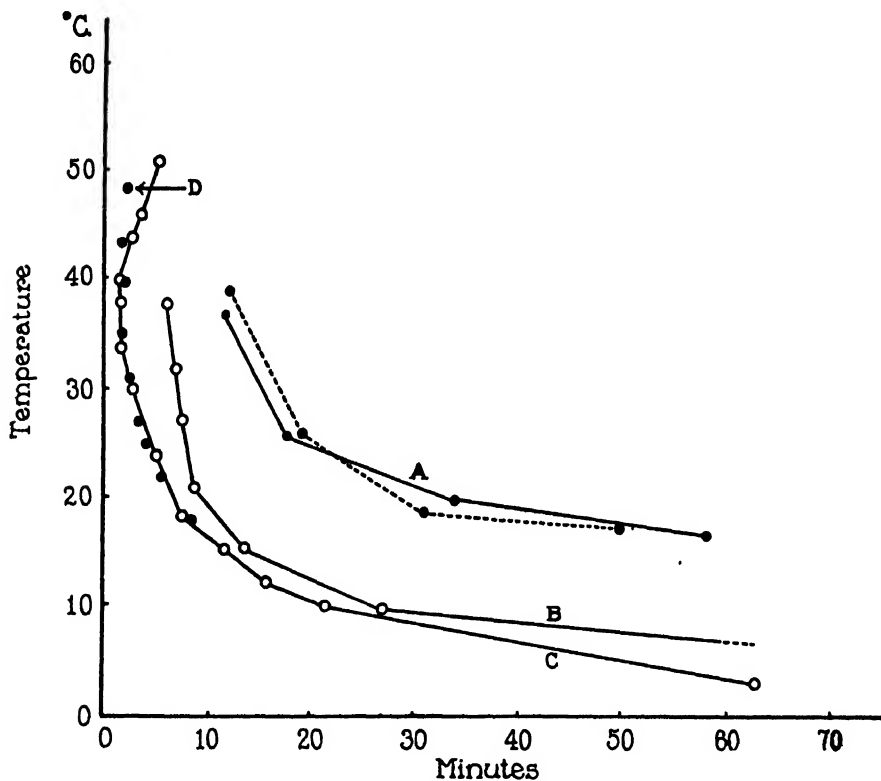


TEXT-FIG. 1.

Technical factors in this procedure which may influence the results are indicated by the following experiment: In each of four rabbits a series of drops from the same wound were tested for clot formation time; also, blood was tested from a fresh cut and from heart puncture. The results are charted in Text-fig. 1. It will be seen that clot formation quickens progressively in blood as it flows from the vein incision, up to a certain limit which may be twice as rapid as that at first. Blood from a fresh cut repeats the first reading obtained, as well as that drawn from the circulation without tissue contact. Thus, under circumstances prohibiting venepuncture, venesection may be used provided the first specimen of blood is taken. Skin puncture alone should not be used, as here the blood flows less rapidly and may be altered from the onset by admixture with "tissue juices."

The temperature at which the specimen coagulates requires control. The effects of temperature change upon the rate of clot formation are shown in Text-

fig. 2 at A. Curves from two rabbits are given, in each of which four specimens, taken simultaneously by heart puncture, were examined at different temperatures, ranging from 17–39°C. Clot formation is seen to vary in rate with the temperature, being greatly delayed at the lower levels. The relation, however, is not strictly direct, for the change in rate per degree change in temperature becomes greater as the scale is descended. Body heat (37.5°C.) is the logical condition for the test but this is too high to be maintained readily by the simple means of heat regulation to be described, and 32°C. has been selected. This temperature

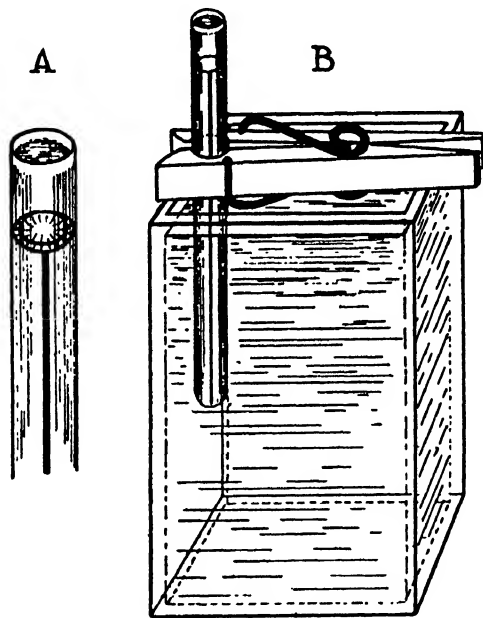


TEXT-FIG. 2.

is sufficiently high that the readings differ but slightly from those at 37.5°C. and, also, are affected inconsiderably by variations in temperature to the extent of 1° or 2° as may occur inadvertently in the technique.

Apparatus.—The articles required are a specimen platform, test-tube and holder, water bath and thermometer (Text-fig. 3). The platform is a thin, concave disc of metal, 1.0 cm. in diameter, supported upon a rod 10 cm. in length. The test-tube is so proportioned as to receive the platform disc readily, is 12 cm. in length and made of clear glass. The test-tube holder is of the type illustrated. A vertical sided jar of about 2 liters capacity serves as water bath.

The parts are assembled for use as follows: The platform disc is coated to at least 1 mm. thickness by dipping in melted paraffin several times, care being taken to preserve the concavity of the upper surface. The tube is filled with mineral oil (Squibb's) to within a few mm. of the top, and into it is dropped the platform, the disc of which will then lie about 1.5 cm. under the oil (Text-fig. 3, *A*). The tube, grasped in the holder, is now suspended part way in the water bath (at about 45°C.) and 5 minutes time is allowed for the column of oil to reach a stationary temperature (Text-fig. 3, *B*). The temperature of the layer of oil overlying the platform is then taken, the thermometer bulb first being warmed in the hand. It is desired to obtain in this section of the oil column a heat level of 32-33°C., and the tube



TEXT-FIG. 3.

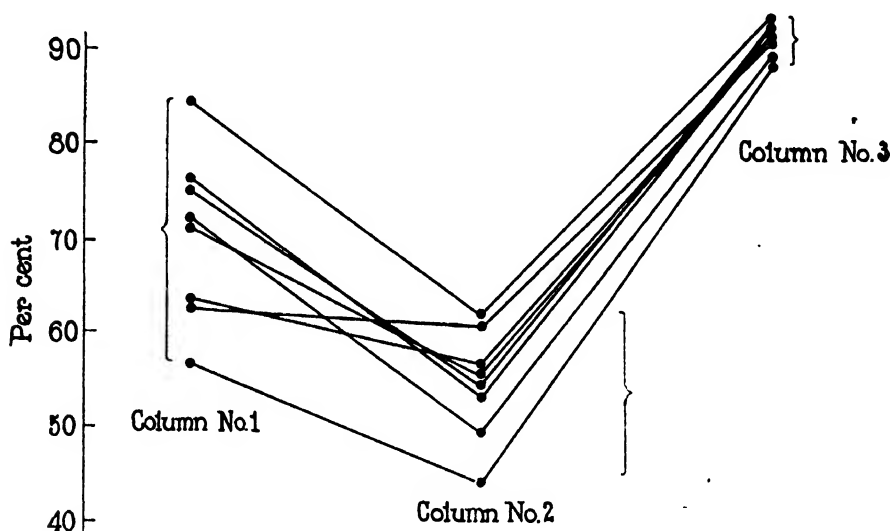
is further lowered or raised in the bath until the temperature is secured. Once adjusted, it will not fall appreciably during the test.

In dealing with a series of animals a rack of test-tubes and a larger water bath have been used. The thermometer is fixed in one tube with its bulb at the level of the platform discs, and by adjusting the height of the rack in the water, and lowering it slightly from time to time as the water cools, all tubes may be kept at the desired temperature for as long as 3 hours while they are being used.

Method.—A small quantity of blood is drawn with a syringe, and, with the point of the needle immediately under the oil, a single drop about 0.6 cm. in diameter is injected and allowed to rest upon the platform, care being exercised that it does not touch the sides of the tube. The size of the drop is unimportant except as the

larger specimens are more easily read. It is observed closely for the first evidence of clot retraction. Fine indentations in the surface indicating this are best seen by bringing the high light to play on the sides of the drop; and warping is detected from above. The interval of time elapsing between securing the blood and the onset of retraction is recorded.

The blood specimen is obtained in the rabbit usually from the ear vein. The skin surface here is shaved, cleansed and dried, then vessel dilatation is produced by applying warmth underneath (small electric light bulb) and a razor cut is made directly into the vein where it lies beneath the skin. Blood will spurt out, and the first that appears is drawn into a syringe or rubber bulb pipette (medicine dropper) for testing.



TEXT-FIG. 4.

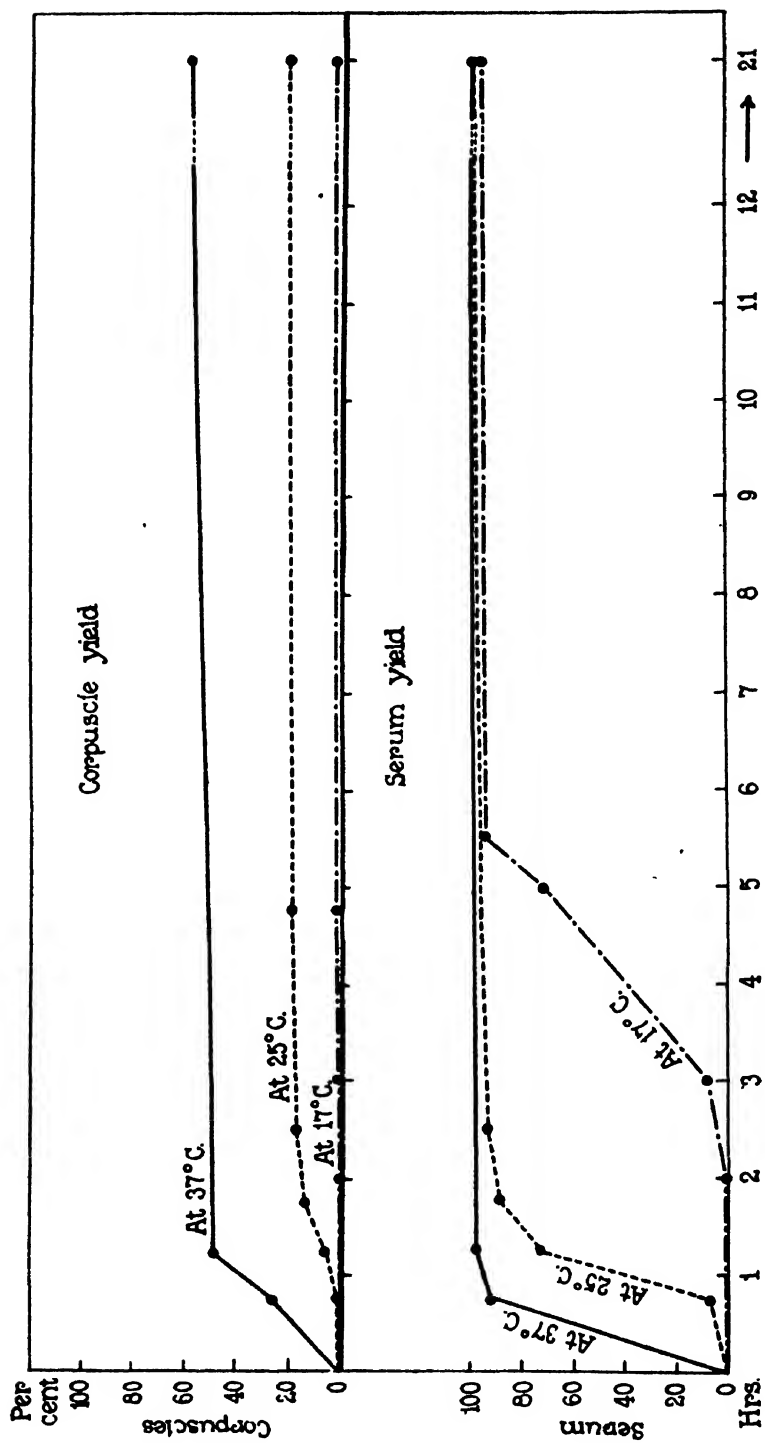
Results.—70, or one-third, of the number of rabbits whose clot formation time at 32°C. has been examined, were normal animals and gave readings between 4 and 8 minutes, averaging 5½ minutes. In those with various diseases the readings varied between 2 and 36 minutes. Incoagulable blood renders no end-point; the drop remains symmetrical and the corpuscles therein sediment, leaving a supernatant fluid of ground glass opacity.

Corpuscle sedimentation occurs also to a degree in certain blood specimens which go on to coagulation. This is accompanied by a prolonged clot formation time, although the latter is frequently found independently. The significance of sedimentation will be discussed below.

II. Clot Retraction.

Experimental Basis.—Extent of Retraction.—Since blood clot retracts with a loss of substance (serum and an amount of corpuscles), it is possible to study the characteristics of clot retraction quantitatively in terms of this substance. For example, if a known amount of rabbit blood be collected and allowed to coagulate completely, it will be found that the material which exudes from the clot amounts to about 70 per cent of the specimen; or, in a series of normal rabbits, between 55 and 85 per cent (Text-fig. 4, Column 1). If these exudates are then centrifugalized, to separate the corpuscles, and the amounts of serum compared, a lesser range of variation will be found (Text-fig. 4, Column 2). But it is logical to suppose that a given specimen of blood can deliver serum only in amount depending upon the available fluid (plasma) content. If, therefore, estimations of the plasma-corpuscle ratios in the blood of these animals be obtained (hematocrit test) and each of the above serum values be expressed in percentage of the plasma content, the range of results will be reduced to within narrow limits (Text-fig. 4, Column 3). It is evident, accordingly, that the normal extent of retraction of blood clot, expressed in terms of the relative amount of fluid lost in the process, is a fairly constant function.

Rate of Retraction.—The rate with which clot retracts, moreover, can be indicated by taking readings of this sort at intervals during retraction. Thus, in Text-fig. 5, the lower chart presents the curves of serum output of three specimens of blood from a rabbit. In order to vary the retraction rate each was placed at a different temperature to coagulate. Marked differences in rate are to be seen, the highest occurring in the specimen at the highest temperature; but the final readings, that is, the ultimate extents of retraction, in the three were the same. For comparison with these curves, the quantities of corpuscles which accompanied these serum yields at each reading during retraction, expressed in the same manner, are given in the upper chart of Text-fig. 5. Here again the curves demonstrate differences in rate, but the final values are not at all similar and vary also with the rate. It would appear that the total amount of corpuscles extruded from blood clot in retracting depends in some manner upon the rate of that process and in no wise upon its ultimate extent.



TEXT-FIG. 5.

Further indication that the total corpuscle output from blood clot is an index of clot retraction rate is to be had in the following experiments, in which clot retraction rate is altered artificially by various means.

Table I contains the data of experiments utilizing the accelerating influence upon coagulation of contact of foreign substances (glass and copper) with the blood. The corpuscle yield was read upon specimens from three rabbits, the blood being contained during coagulation in paraffin-lined tubes and covered with

TABLE I.
The Accelerating Effect of Foreign Body Contact on Retraction Rate.

	Corpuscle readings		
	Rabbits		
	A	B	C
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Specimen 1 (tube paraffin-coated and with oil)	6	15	47
Specimen 2 (tube omitting paraffin coat)	17	27	
Specimen 3 (tube paraffin-coated and with oil, added piece of bare copper wire)			74

TABLE II.
The Retarding Effect of Neutral Foreign Bodies on Retraction Rate.

	Corpuscle readings
	<i>per cent</i>
Specimen 1 (tube paraffin-coated and with oil)	47
Specimen 2 (the same as above; added one piece paraffin-coated copper wire)	28
Specimen 3 (the same as above; added three pieces paraffin-coated copper wire)	23

oil. With these as controls, readings of other specimens were made treated in all respects similarly, except that in two instances the paraffin lining was omitted, and in the third a fragment of copper was placed in the blood. The corpuscle yields in all of the latter were distinctly greater than in the controls.

The experiment in Table II made use of the retarding effect upon clot retraction of mechanical obstruction to the process. In comparison with a control reading, obtained as described in the above experiment, two other specimens from the same animal were tested in which there had been placed strands of copper

wire rendered neutral to coagulation by a coating of paraffin. The corpuscle reading from each of the latter was less than from the control and diminished in proportion to the number of wire strands present.

In Table III data are given concerning the retardation of clot retraction by exposure to air. Control readings were made as before in three rabbits and, for comparison, specimens were read from which the oil covering had been omitted. The corpuscle output was thereby lowered.

Agitation of the blood retards coagulation. In comparison with the usual controls in two rabbits (Table IV), specimens were tested similar to the others in all

TABLE III.

The Retarding Effect of Exposure to Air on Retraction Rate.

	Corpuscle readings		
	Rabbits		
	A	B	C
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Specimen 1 (tube paraffin-coated and with oil)	65	59	24
Specimen 2 (tube omitting oil)	38	45	14

TABLE IV.

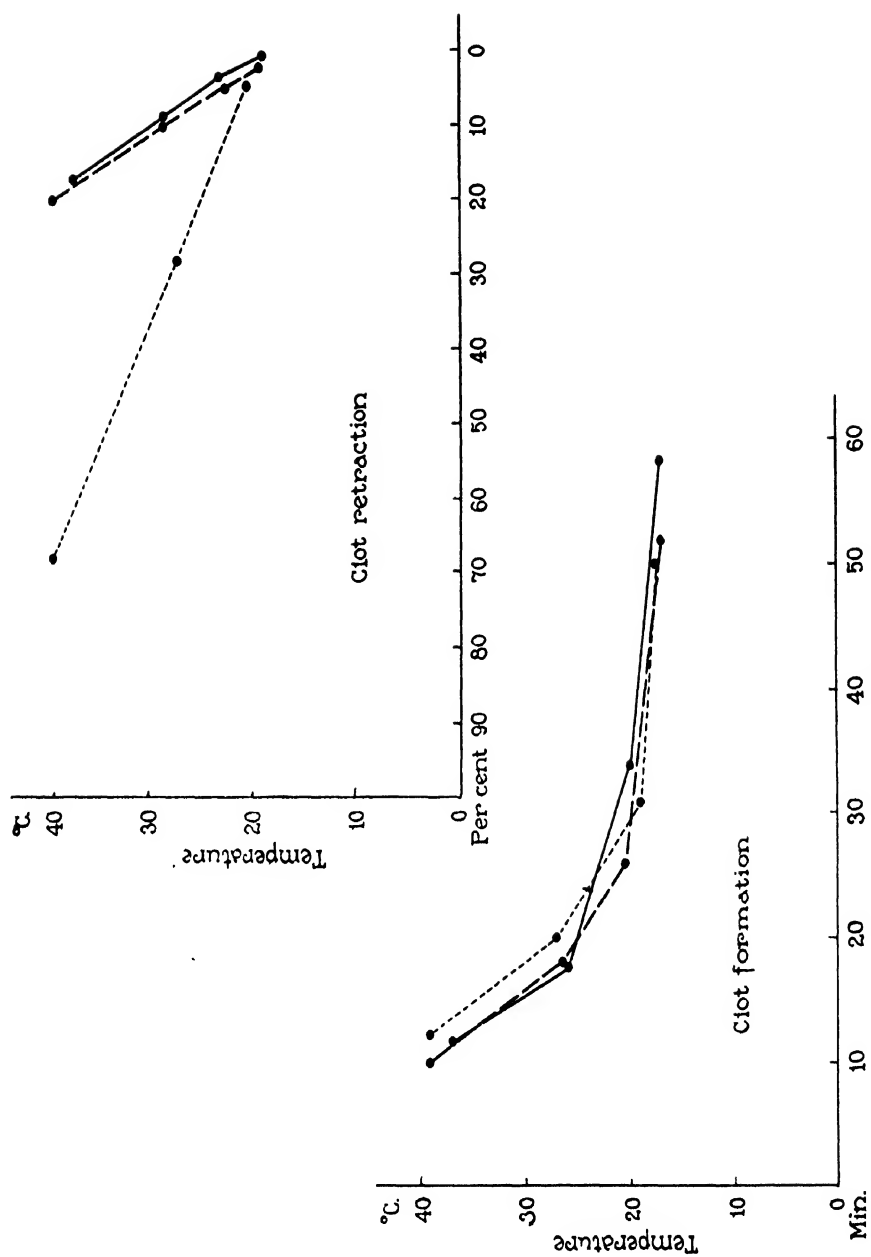
The Retarding Effect of Agitation on Retraction Rate.

	Corpuscle readings	
	Rabbits	
	A	B
	<i>per cent</i>	<i>per cent</i>
Specimen 1 (tube paraffin-lined and with oil)	47	31
Specimen 2 (the same as above)	49	
Specimen 3 (the same as above, stirred 1 min. after shedding)	29	23
Specimen 4 (the same as above, stirred 2 min. after shedding)		16

respects except that shortly after shedding the blood was slightly agitated. This had the effect in each instance of reducing the corpuscle yield.

Still further and surer proof of the significance of the corpuscle reading as an index of clot retraction rate is had from experience with the value in disease, for wherever retraction was found retarded as indicated by abnormally low intermediate serum readings, the total corpuscle yield was always depressed.

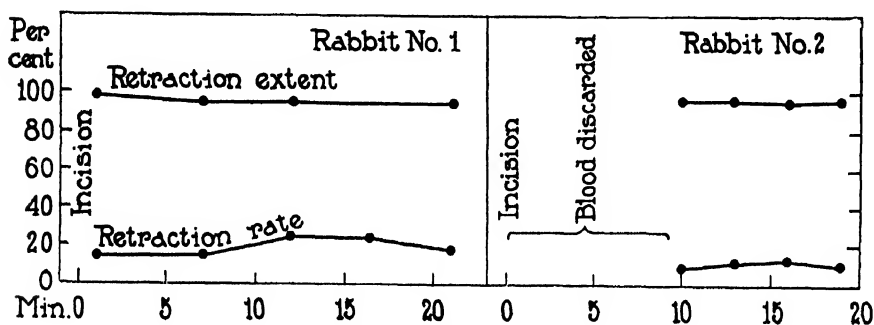
The means is presented, accordingly, of estimating the rate and the extent of blood clot retraction: The total fluid yield from clotted blood is separated into its serum-corpuscle fractions and, properly corrected, the corpuscle value indicates the rate and the serum value the extent of clot retraction.



TEXT-FIG. 6.

Relation of Clot Retraction to Clot Formation.—We may proceed to examine the behavior of clot retraction in its relationship to that of clot formation.

In Text-fig. 6, on the right, are curves of retraction rate secured from blood of three rabbits. In each instance four specimens were taken and allowed to coagulate at temperatures extending from 17–38°C. It will be seen that not only does rate vary with temperature but the effect is proportional, *i.e.*, each curve is virtually a straight line. In Text-fig. 6, on the left, are curves of clot formation rate from these animals secured under the same conditions of temperature (see also Text-fig. 2, A). Here the relation between rate and temperature is represented by a line of hyperbolic form. Moreover, while the formation rates at corresponding levels are nearly alike in the three animals, one of them gave a retraction rate widely separated from those of the other two. Apparently, clot retraction and clot formation behave quite independently in rate.

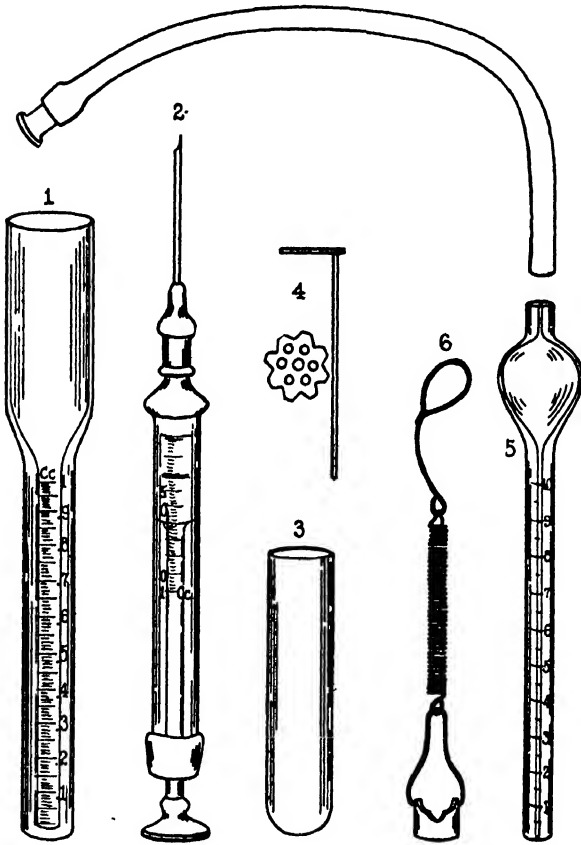


TEXT-FIG. 7.

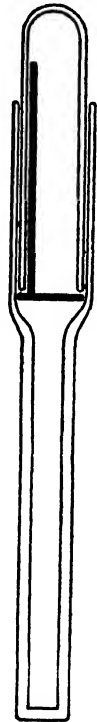
This individuality of rate of the two phases of coagulation may be demonstrated in another way: Five specimens of blood from an animal were allowed to coagulate at different temperatures, thus, Nos. 1 and 2 at 17°C., No. 3 at 25°C. and Nos. 4 and 5 at 37°C. As coagulation proceeded, Specimens 2 and 4 were observed closely and at the completion of clot formation they were interchanged, *i.e.*, No. 2 was placed at 37°C. to undergo retraction, and No. 4 at 17°C. After coagulation was complete, the yields from all specimens were read (Fig. 2). The corpuscle yields from Nos. 1, 3 and 5 varied as would be expected with the temperature; the corpuscle yield of No. 2 was a duplicate of that of No. 5, and that of No. 4 was a duplicate of No. 1. Evidently, specimens which retracted at the same temperature retracted at similar rates, without reference to the temperature (rate) at which clot formation took place.

It has been demonstrated (Text-fig. 1) that clot formation is markedly accelerated by contact of the blood with the tissues. In Text-fig. 7 are given clot retraction readings in two rabbits, the specimens being taken successively from the same wounds. The curves are practically horizontal. From this it is evident that clot retraction is not affected as is clot formation by tissue kinase.

*Apparatus.*¹—Record syringe (Text-fig. 8, No. 2), of 1.2 cc. capacity, graduated in 0.2 cc. divisions. Test-tube (Text-fig. 8, No. 3), of 1.0 cm. internal diameter and 5.0 cm. length. Graduated centrifuge tube (Text-fig. 8, No. 1), of 1.0 cc. capacity. Separating disc (Text-fig. 8, No. 4), which is a perforated metal disc of the same diameter as the test-tube. Hematocrit (Text-fig. 8, Nos. 5 and 6), described elsewhere (10, 11).



TEXT-FIG. 8.



TEXT-FIG. 9.

In preparation for the test the glassware is carefully cleaned, particular attention being paid the syringe that it contains no residue of blood and is dry. The test-tube is given a heavy lining of paraffin, thus: It is dipped into melted paraffin

¹ These instruments may be obtained from the Gottlieb Greiner Company, New York City, with the exception of the hematocrit which is manufactured by the Arthur H. Thomas Company, Philadelphia, and Firma Arno Haak, Jena, Germany.

which has been cooled almost to the point of thickening, and on being removed is immediately placed inverted under cold running water. The external surface is then wiped free of paraffin and the inner surface examined to make sure that it has been coated entirely and is free from water.

Method.—The syringe is filled to 1.2 cc. with blood (for the rabbit see technique of collecting blood above) and 1.0 cc. of this is deposited precisely in the bottom of the test-tube (thus is avoided error from the dead space air bubble in the syringe). A few drops of mineral oil are then added to cover the specimen and it is placed at 37°C. for coagulation.

An hematocrit test is now performed.

From 5 to 6 hours after collection of the specimen, the test-tube is removed from the incubator, covered with the separating disc and inverted into the centrifuge tube (Text-fig. 9). This is then centrifugalized for 30 seconds at the lowest speed (about 500 R.P.M.) necessary to displace the fluid contents of the test-tube completely through the separating disc into the graduated tube. Now, the test-tube, separating disc and blood clot are removed, and the graduated tube, containing the fluid yielded from the clot, is centrifugalized at high speed (about 3000 R.P.M.) for 5 minutes to separate corpuscles from serum.

The amounts of corpuscles and serum are read from the scale on the centrifuge tube (Fig. 2), and these values are corrected with reference to the hematocrit result, to give the final readings, as follows:

If x = final serum reading, representing retraction extent,

y = final corpuscle reading, representing retraction rate,

a = serum reading from tube; for example, 0.56 cc.,

b = corpuscle reading from tube; for example, 0.18 cc.,

and c = amount of corpuscles in whole blood; for example, where the hematocrit reading is 40 per cent, the corpuscle content of the specimen is 0.40 cc.,

then $x = \frac{a}{1.00 - c}$ for example $x = \frac{0.56}{1.00 - 0.40} = 0.93$ (read 93 per cent),

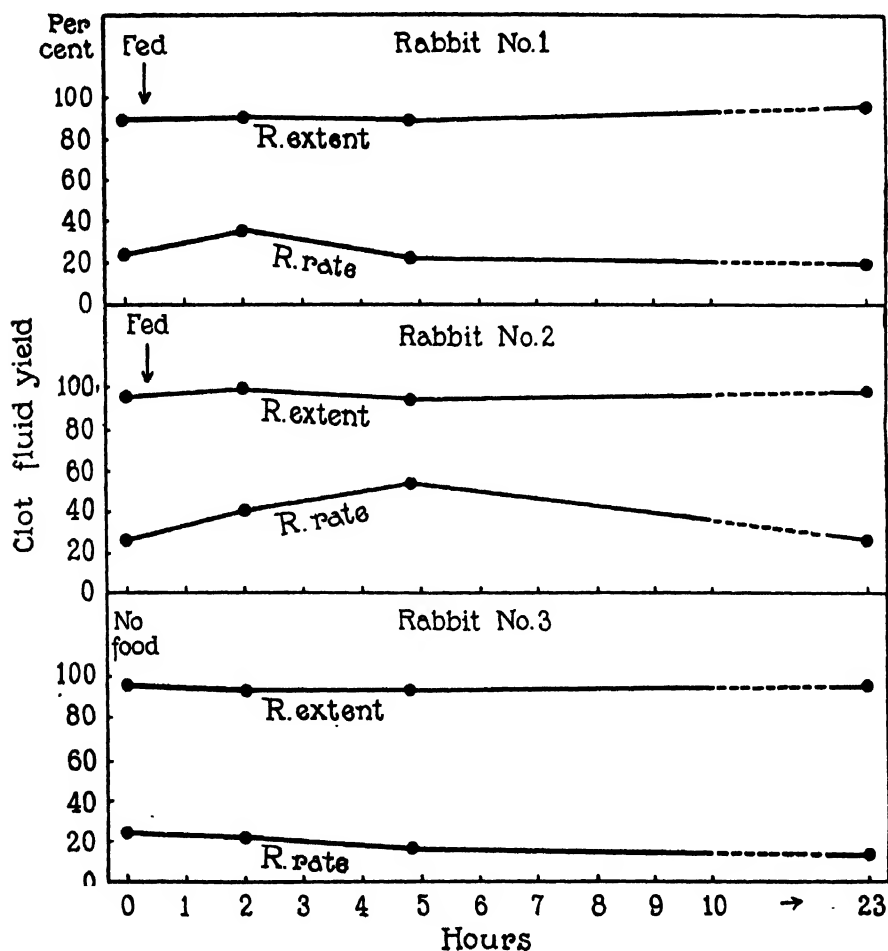
and $y = \frac{b}{c}$ for example $y = \frac{0.18}{0.40} = 0.45$ (read 45 per cent).

Certain technical points require emphasis:

It is of first importance in securing the specimen that no more than 20 seconds shall elapse between its leaving the circulation and entering the test-tube, for agitation of the blood in the early stages of coagulation produces marked reduction in coagulation rate and renders the reading misleading (Table IV). This small quantity of blood may be obtained rapidly enough when withdrawn by venepuncture, but in the rabbit where it is collected from the pool of blood issuing from an incision the task is often difficult. When there is any question about the rapidity of collection, a second specimen should be taken and, if the reading is higher, adopted. Once the blood is in the tube further haste is not imperative, although no more than 2 minutes should intervene before placing it in the incubator.

If hemolysis occurs and the serum is found tinted, no reading is to be made, since bacterial activity has probably altered the specimen. For this reason the incubation is limited to 6 hours.

Where it is obvious from inspection after incubation that clot retraction has occurred to a slight extent only, it is better to remove the accumulated serum by pipette for measurement rather than by centrifugalization, for such clots are soft



TEXT-FIG. 10.

and give up additional fluid readily on pressure. Where clot formation fails or takes place only in portions of the specimen, it is of course impossible to measure clot retraction and fluid readings become valueless. In these cases there will usually be found upon the separating disc either no clot at all or misshapen clot fragments; even where the clot appears normal the condition may be detected,

since the serum reading is abnormally low (under 87 per cent) and the corpuscle value high (over 30 per cent). Such a result is, of course, indicative of an exceedingly defective coagulation and is of value qualitatively.

At the time of examination the subject should be fasting. The apparent accelerating effect of food ingestion upon clot retraction is illustrated in Text-fig. 10.

Results.—Records are at hand of 810 readings of clot retractility. The normal rate of clot retraction lies between the values 40 and 60 per cent (corpuscle output) and the normal extent between 87 and 100 per cent (serum output). In disease, instances were found of all grades of depression of these values, to 0 per cent in non-retractile blood clot.

The margin of error from various technical sources allowed in the interpretation of readings is 10 per cent of the rate value and 2 per cent of the extent value.

DISCUSSION.

The factor here measured as clot formation rate is but another expression of the same function of the blood coagulative process as the clotting rate values of other methods. This is shown by their similarity of behavior in the presence of various disturbing influences, such as tissue kinase and foreign body contact with the blood, but more particularly by the type of their reaction to temperature change. Thus, the temperature curve of clot formation as given in Text-fig. 2, at *A*, runs parallel with those at *B*, *C* and *D*, which are constructed from data published respectively by Lee and White (7) with the tube inversion method of Howell, by Addis (6) with his modification of the hanging drop method of Brodie and Russell, and by Dale and Laidlaw (12) with their shot tube procedure. However, judging from the positions occupied by the curves relative to each other, the end-point of clot formation is situated further along in the process of coagulation and would logically have the advantage of magnifying changes occurring in coagulation rate. In this procedure, also, the conditions under which the specimen resides while coagulating are nearly neutral, and the rate obtained may be considered as practically basic for the blood examined.

Sedimentation of corpuscles in the drop of blood tested is a phenomenon especially to be noted. It has been observed in specimens of coagulating blood by others, and is the cause of production of the

"buffy coated" blood clot (13, 14). Addis (15) found it associated with prolonged clotting time in extremely ill, septic patients. Lee and White (7) noticed it in various diseases, and so frequently was it accompanied by delayed clotting time, that the phenomenon was used as confirmatory evidence of the latter condition. Sedimentation has been found here also followed by an extended end-point of clot formation, but it probably has particularly to do with the beginning-point and indicates delay in the onset of coagulation. This can be shown as follows: If a specimen of blood is chilled directly after collection, the corpuscles will soon commence to gravitate and this will continue until the temperature is elevated and coagulation is allowed to proceed; should, however, the chilling be postponed for 1 minute after shedding, to allow coagulation to begin, and is then instituted, sedimentation will not take place much as the clotting end-point may be delayed by the low temperature. Also, it has been found that, where corpuscle sedimentation occurs in disease, examination of the blood by means of the wire loop of Buckmaster (5) demonstrates marked delay in the appearance of certain alterations in the specimen peculiar to the onset of coagulation. In the gravitation of the corpuscles, accordingly, is presented a means of detecting retarded initiation of coagulation, a factor in addition to the estimation of clot formation rate.

Clot retraction is a function which depends upon physical principles quite separate and distinct from those underlying clot formation, since, as has been demonstrated, they behave differently in the presence of various disturbing influences.

It has been necessary to resort to indirect means for the estimation of clot retractility. Direct measurement of size alterations in the clot itself with any degree of accuracy is well nigh impossible, since the pattern of retraction is extremely variable, particularly in the early stages, and measurement in one or two diameters alone is not sufficient. However, the amount of fluid yielded from the clot during retraction is as logical a measure and more readily applied; either procedure must take into consideration the plasma-corpuscle ratio of the blood and the amount of corpuscles extruded with the serum, in order to narrow the range of normal values and distinguish the abnormal. The reason is not apparent for the parallelism found between the rate of clot retraction and the quantity of corpuscles which escapes from

the clot. This has probably something to do with the mechanics of fibrin retraction. In early tests, an additional serum reading was made 1 hour after collecting the blood and compared with the final serum reading as an indicator of clot retraction rate. But this was abandoned when the significance of the corpuscle output was appreciated, and, not only has the technique been simplified thereby, but the corpuscle reading has proven much more delicate in determining changes in rate.

SUMMARY.

The gross phenomena of normal blood coagulation have been studied for the purpose of obtaining methods for estimating certain characteristics of coagulation in connection with a malignant tumor of the rabbit. The technique is described for the estimation of clot formation rate and of clot retraction rate and extent. The range of normal and abnormal values secured by these procedures is given.

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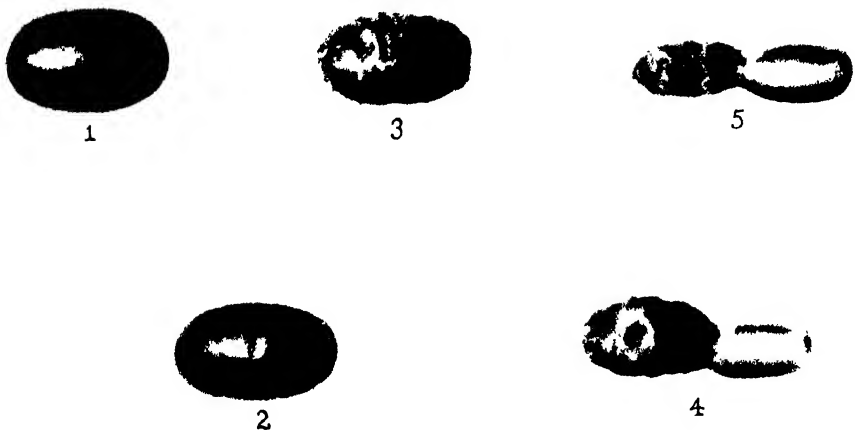


FIG. 1.

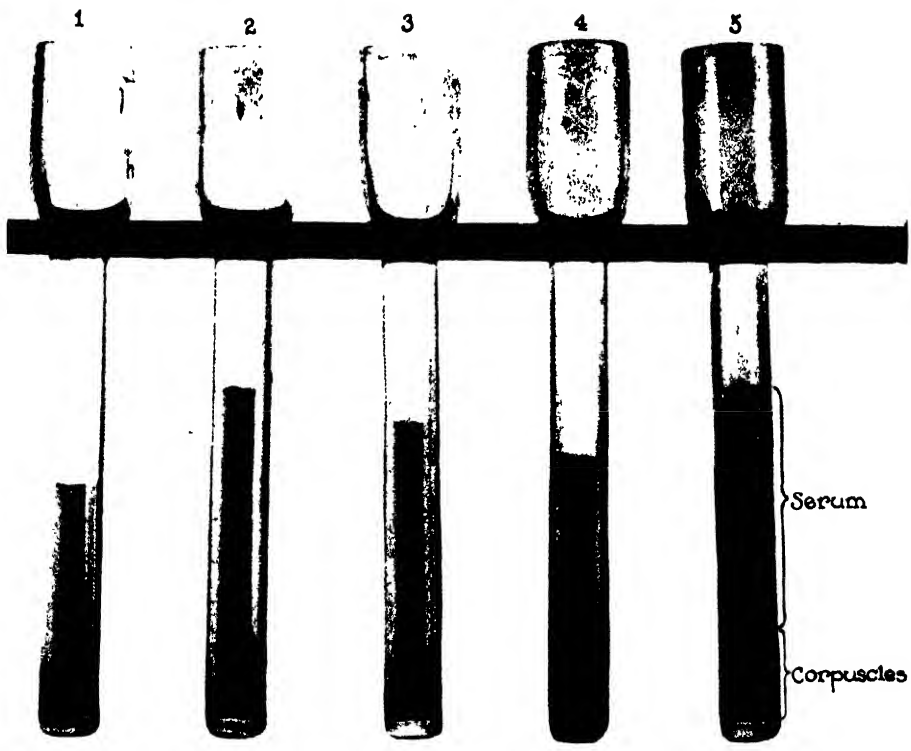


FIG. 2.

(Van Allen: Blood coagulation. I.)

STUDIES IN BLOOD COAGULATION.

II. BLOOD COAGULABILITY IN MALIGNANT TUMOR AND OTHER DISEASES OF THE RABBIT.

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(Received for publication, August 23, 1926.)

Alterations in the coagulability of the blood will be described which occur in various stages of the growth and extension of a malignant tumor of the rabbit, and, as a basis of interpretation, there is explained also the behavior of coagulation in other diseases and abnormal states of this animal. The methods of test employed have been described (1), and include estimation of clot initiation and clot formation rate and the rate and extent of clot retraction.

I. Disease in General.

Relation of Blood Coagulability to Presence and Extent of Disease Lesions.

Experimental.—100 adult male rabbits, taken from stock as received from the breeders, were placed under observation for 1 or more weeks.¹ All grades of physical condition were presented, although the great majority were free from external evidences of disease. The blood coagulability of each animal was then tested, and autopsy examination was made within 24 hours afterward. A comparison of the readings with the anatomical findings follows.

Results.—As regards clot formation rate, the readings in almost every instance were within the limits of normal variation, *i.e.*, between 4 and 8 minutes. Five exceptions to this presented, and all of these were in extreme stages of disease: Two gave clot formation time of 10 minutes, and showed at autopsy fatty degeneration of the kidneys and profound cachexia, which changes were probably secondary to an occult disease process. One rabbit with extensive coccidiosis of the

¹ These rabbits are part of a larger group being used for other experiments in the study of normal and abnormal organ weight variations.

liver, another with snuffles complicated by septicemia and a third markedly emaciated but without discernible lesions gave delayed clot initiation and an irregular clot formation allowing of no distinct reading.

The extent of clot retraction was also normal in the majority of the animals, *i.e.*, reading between 87 and 100 per cent. In the last

TABLE I.

Autopsy finding		Analysis of groups on a basis of clot retraction rate				
		40-60	30-40	20-30	0-20	No reading obtainable
		per cent	per cent	per cent	per cent	per cent
Rabbits grouped on a basis of stress of disease	11, without lesions and in good physical condition.....	91	9			
	10, with healed lesions of various types.....	80	20			
	24, with coccidial lesions, small and of questionable activity.....	25	33	29	13	
	13, in process of recovery from various types of infections.....		32	60	8	
	18, with purulent infections, snuffles, mastoiditis, etc.....		5½	11	78	5½
	8, with coccidial lesions, numerous and obviously active.....			40	50	10
	4, emaciated, with fatty degenerative lesions of kidneys.....			25	75	
	2, emaciated, with active parasitic lesions of kidneys.....				100	
	2, emaciated, with focal lesions of appendix.....				100	
	3, emaciated, but without discoverable disease process.....				67	33

three rabbits above mentioned occurred the only abnormal values, 85, 85½ and 81 per cent, respectively.

Clot retraction rate, on the other hand, was extremely variable, both in rabbits obviously ill and those without symptoms. The autopsy findings are correlated statistically in Table I with the retraction rate values. On the left, the rabbits are grouped according

to the type of disease found and the groups are arranged in order on a basis of degree of physical deterioration (disease stress) evident. On the right, analysis is made of each group from a standpoint of the clot retraction rates, and for this purpose four divisions are used. It will be seen that in the highest rate division, 40 to 60, fall 91 per cent of animals without lesions and in good physical condition, also, 80 per cent of those with healed lesions only and 25 per cent with insignificant coccidial lesions. As analysis proceeds thus from group to group dealing with disease of increasing severity, the distribution of rate values plainly shifts across the scale, and the last five groups lie entirely within the lowest rate division, 0 to 20. The animals which fall principally in the intermediate divisions, 20 to 30 to 40, are those with moderately active disease or in process of recovery from severe infections. Retraction rate could not be measured in three rabbits, those last referred to above, because of the fragmentary nature of the coagulative process.

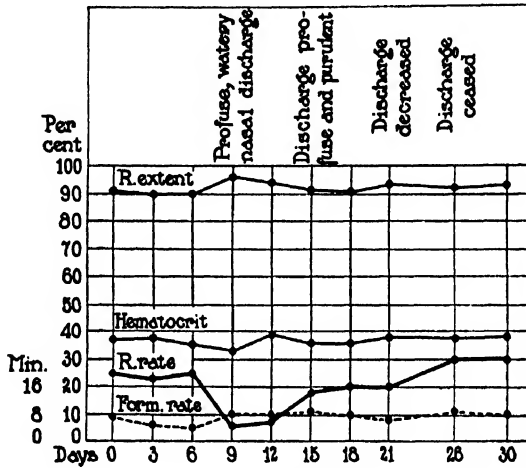
Five rabbits are excluded from the table for special mention: Two had been inoculated intratesticularly with the spirochete of yaws and had an early, active orchitis. At autopsy no other lesions were found. The retraction rates were normal, 42 and 48 per cent. Two rabbits had well developed goiter, were otherwise in good condition and gave readings, 45 and 56 per cent, within the normal range. One rabbit although a female was included in the series to illustrate a point of interest. She was abundantly fat and sleek and presented nothing pathological beyond a small, healing *cuniculi* papule on the genitalia. Nevertheless, the clot had an extremely low retraction rate, 9 per cent; at autopsy pregnancy at about the 10th day was discovered.

Relation of Blood Coagulability to Course of Disease.

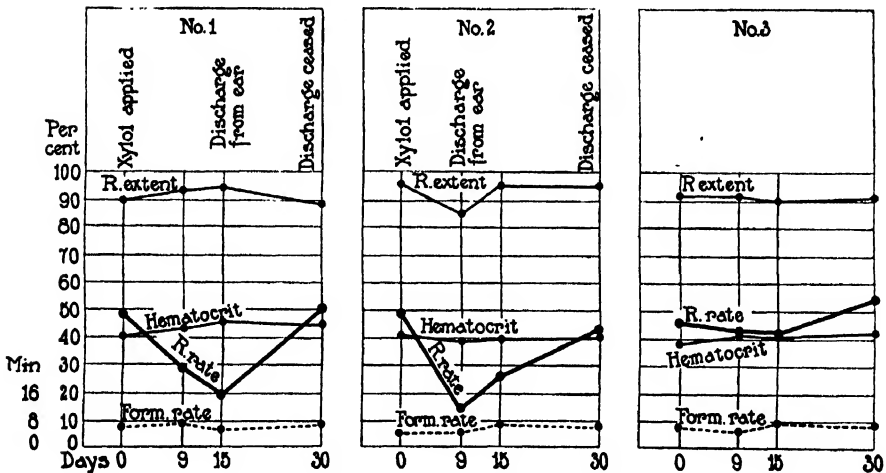
Experimental.—The behavior of blood coagulation was investigated during the course of various abnormal and diseased states, including instances of spontaneous infections, non-bacterial lesions and artificially induced affections of the blood coagulative mechanism. In each case, periodic tests were made and symptoms recorded, with autopsy examination at the termination of the experiment.

Findings.—Brief protocols of typical examples of each disease are given:

Snuffles.—Text-fig. 1 is the chart of a rabbit which had been followed for some time because of chronic snuffles. The infection persisted for about 2 months, gradually cleared, and the nasal discharge had ceased, when abruptly a relapse set in with profuse discharge and sneezing and the animal passed through a



TEXT-FIG. 1.

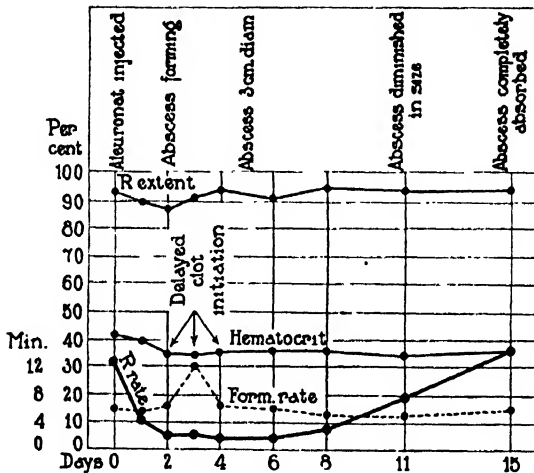


TEXT-FIG. 2.

second typical course of the disease, this time lasting 3 weeks. At autopsy, the nasal passages were free from inflammation; the right middle ear spaces contained a small quantity of inspissated pus. There was no other disease. Evidently the rabbit had had chronic otitis in addition to the attacks of snuffles. The period of snuffles remission only is included in the chart of blood findings. The rate of

retraction had been distinctly below normal and dropped still farther with the redevelopment of rhinitis. Later, as the infection cleared, the curve rose to its previous level. The readings of retraction extent and clot formation rate showed throughout no variations beyond the normal range.

Auditory Canal Abscess.—The charts are given in Text-fig. 2 of three rabbits from a group of fifteen which were being followed as controls for other purposes. At this time xylol was being used routinely painted on the inner surface of the ear to produce vessel dilatation for the collection of blood specimens, and in two instances, Rabbits 1 and 2, the xylol extended accidentally into the auditory meatus. The applications were discontinued immediately in favor of other methods, but the inflammation in the canal persisted and an abscess developed in its recesses. Rupture occurred in 2 weeks, with drainage of pus for another week. Rabbit 3 is given for comparison, for here no irritation arose, aside from a



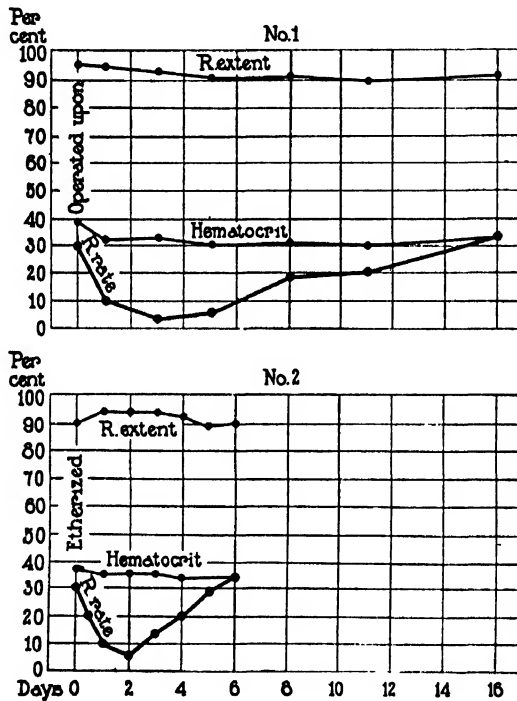
TEXT-FIG. 3.

slight erythema of the pinna. From the charts, it is to be seen that in the cases of the first two rabbits clot retraction rate fell after xylol application to a low level on the 15th and 9th days respectively, and with rupture of the abscess the reading began to rise and reached normal with the healing of the lesion. Retraction extent in the first rabbit was elevated in the early stage of inflammation, but in the second, where the rate drop had been more sudden and pronounced, the extent reading also fell temporarily. Both rate and extent of clot retraction in the third rabbit remained during this period within the normal limits. Clot formation showed no abnormalities in any of the animals.

Subcutaneous (Sterile) Abscess.—Six rabbits were injected either subcutaneously or intrapleurally with 0.5 cc. of a 10 per cent suspension of aleuronat. In each, pus accumulated at the site of injection, and in two of the animals which were allowed to live for 3 weeks the abscess was absorbed and no trace remained except

slight local increased vascularity. The record of one of the latter is represented in Text-fig. 3, the two being similar in all respects. The curve of retraction rate fell sharply within 24 hours after injection and remained at a low level for 6 days during the period of abscess development. Then the curve began to rise and by the 15th day was at normal, when the lesion was healed. The extent of retraction did not leave the normal range. Clot initiation and formation were delayed slightly at the height of the inflammatory reaction.

Operative Incision.—Four rabbits were operated upon under ether anesthesia with sterile precautions for the purposes of other work. In two the spleen was removed and in the others laparotomy only was done, as control. The wounds



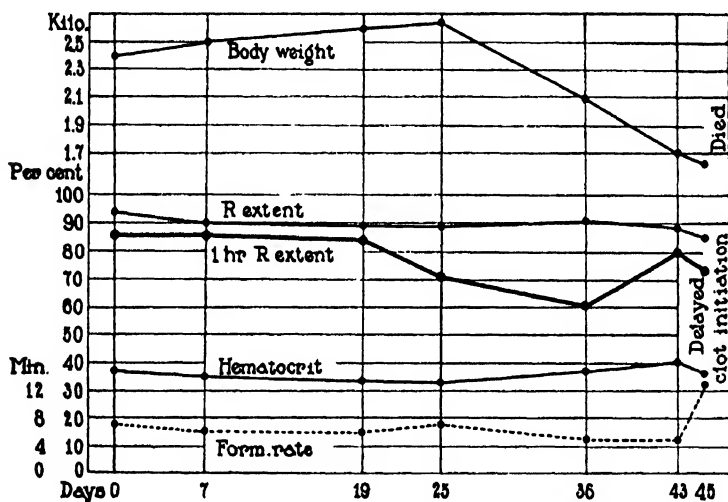
TEXT-FIG. 4.

healed *per primam*. The coagulation curves of one of the control rabbits, which were typical of all, are given in Text-fig. 4, No. 1. Immediately after the operation the rate of retraction dropped and reached a low point on the 3rd day, when it began to return and attained its original level on the 16th day. The extent of retraction remained stationary throughout. No clot formation readings were made.

Ether Anesthesia.—In order to ascertain the part played by ether in the preceding results, two animals were anesthetized for a period of $\frac{1}{2}$ hour. The subsequent behavior of clot retraction in both was the same; the curves of one are given in

Text-fig. 4, No. 2. The rate became retarded in a manner similar to that in the chart above, but returned much more rapidly to normal.

Nephritis.—The chart is given in Text-fig. 5 of a normal rabbit which developed nephritis spontaneously while under observation. The animal had been in good health and gained steadily in weight until the 4th week of observation, when it began to grow thinner without other symptoms. 1000 gm. were lost before death 3 weeks later. At autopsy, the body tissues were found dehydrated and wasted; the only lesion presenting was with reference to the kidneys. These organs were markedly swollen, pitted and intensely yellow, with a fine surface stippling. On section, the cortices were found widened, of an opaque yellow color and without architectural markings. The medullæ were pale and presented radial grey streaks. From the chart, it will be seen that clot retraction rate



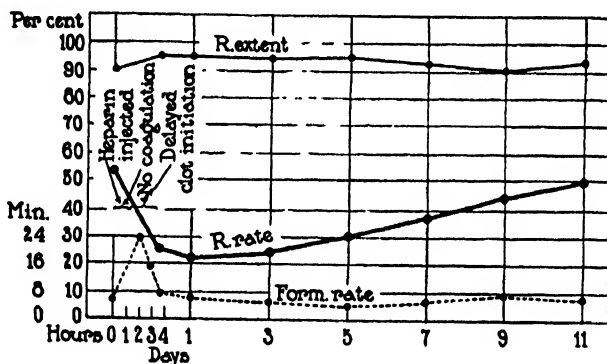
TEXT-FIG. 5.

(estimated by a 1 hour serum reading in comparison with the final serum output, a less sensitive index) showed marked retardation on the 25th and 36th days, a return toward normal on the 43rd day, and another drop before death. Retraction extent and formation rate showed no abnormalities until the final reading, when the latter was delayed.

Specific Affections of the Blood-Coagulative Mechanism.—Effort was made to reproduce experimentally conditions of blood coagulability imitating those in the hemorrhagic diatheses, hemophilia and thrombocytopenic purpura.

The properties of the blood in hemophilia are said to be obtained temporarily in animals by the injection of heparin (2), a phosphatide extracted from the liver by the process of Howell, that is, clotting time is prolonged but, when coagulation ensues, the clot retraction appears normal. In the present study seventeen rabbits

were used and heparin,² at a concentration of 0.5 per cent in physiological saline solution, was injected intravenously in amounts from 5 to 40 mg. per kilo body weight. Both single and series of doses extending over long periods were employed. In none of the animals appeared symptoms of any sort referable to the drug, nor did microscopic examination of the bone marrow, spleen, liver and other organs reveal changes. Marked alterations in blood coagulability were presented however, shown typically in Text-fig. 6. Here a single dose of 17 mg. per kilo was injected. The blood, examined immediately and again after 1 hour, failed to coagulate. 2 hours after treatment a specimen gave moderately delayed initiation of coagulation and formation time of 23 minutes. At 2 hours, 40 minutes, these two functions were still tardy but became normal 1 hour later. Although clot retraction was observed to occur in portions of specimens as early as 2 hours after injection, the process was so imperfect as to exclude estimation. At 3 hours, 40 minutes, the first measurement was obtainable and gave a considerably reduced



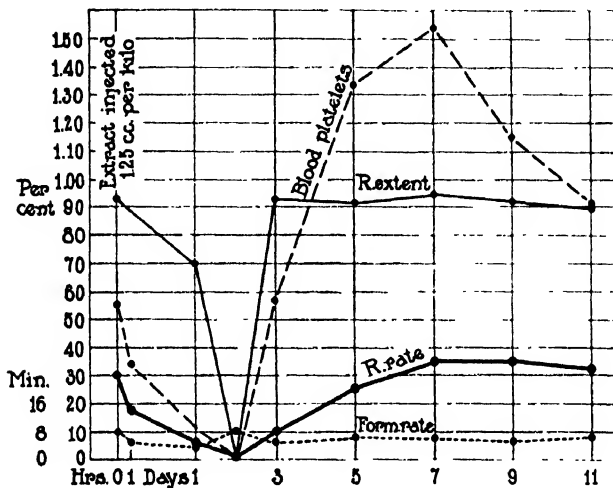
TEXT-FIG. 6.

rate and a normal extent. Further depression in rate was found after 24 hours, and then this function returned gradually to its original level by the 11th day. The other factors of coagulation were normal after the initial interference. These alterations in the blood varied in degree among the animals with the dose employed. After repeated treatments (20 in 60 days, of 40 mg. per kilo) rabbits appeared to have become somewhat resistant to the drug, although this was not consistent.

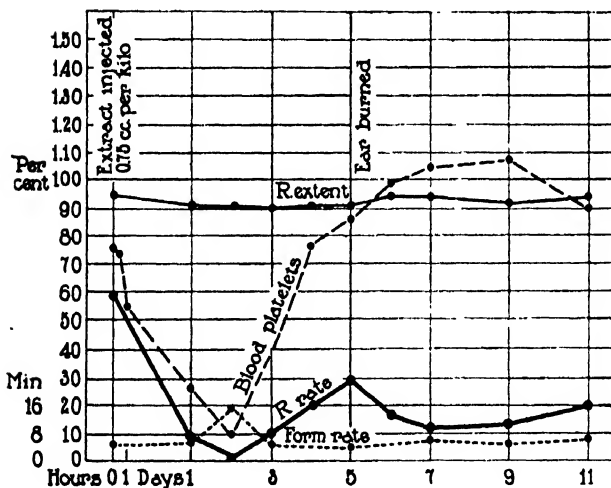
The pneumococcus extract of Avery and Neill (3) is known to have a specific lytic effect upon the blood platelets and to produce in mice a transitory purpuric condition. I am indebted to Dr. Avery and Dr. Julianelle of The Rockefeller Institute Hospital for a supply of the extract. This was first warmed to remove the hemolysin, and amounts from 0.25 to 3.50 cc. per kilo body weight were

² Heparin may be obtained from Hynson, Westcott and Dunning, of 150 Nassau Street, New York City, and Baltimore, Md.

injected intravenously in twelve rabbits. No symptoms were observed, although one rabbit receiving the highest dose was found dead after 18 hours. No purpura or tendency to bleed (as indicated by the incisions in the ear) was evident, even



TEXT-FIG. 7.



TEXT-FIG. 8.

when the platelets were practically absent from the blood stream. Microscopic study of the bone marrow, spleen, liver, lymph nodes and other organs and tissues discovered no changes. Charts of two of the rabbits are given (Text-figs. 7 and 8). Curves have been added representing the platelet content of the blood; these values

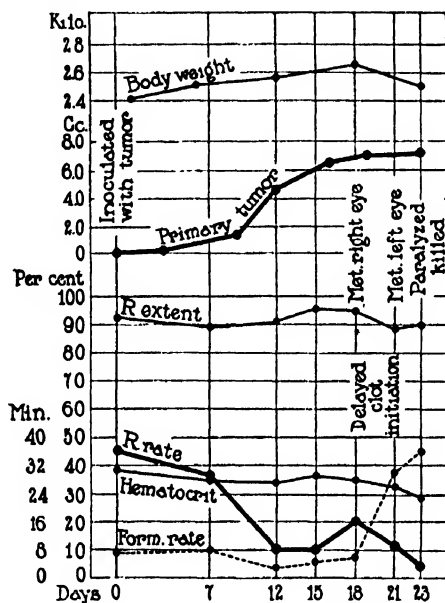
were obtained with the thrombocytocrit,³ an instrument recently described (4), and give estimations of platelets volumetrically instead of numerically as customary. It will be seen that platelet lysis commenced immediately after injection of the extract and reached an extreme degree on the 2nd day. The platelets then reaccumulated rapidly and in great excess, attaining a maximum between the 7th and 9th days, after which their volume returned to normal. This behavior is similar to that described in the mouse by Julianelle and Reimann (5). In Text-fig. 7, following the injection of 1.25 cc. extract per kilo, the rate and extent of clot retraction began to fall within 1 hour, and continued to descend until the 2nd day, when the clot, although of firm consistency, failed to retract. The next day retraction had returned to normal in extent but took place at a very slow rate. The rate returned to its original level by the 7th day. Clot formation showed no departure from normal throughout. In Text-fig. 8, following the injection of a smaller dose, the retraction rate fell as before but its extent remained unaffected. By the 5th day the rate had begun to ascend, but once more was depressed coincidentally with the occurrence of a slight accidental burn of the rabbit's ear while warming it to secure blood, and the typical reaction to the extract was thus altered. Clot formation became slightly retarded on the 2nd day. These changes in blood coagulation were found to vary in degree among the rabbits approximately with the size of dose employed. Even with as small an amount as 0.25 cc. per kilo retraction rate was markedly reduced, but the extent of retraction was affected only with the larger amounts of the extract. A close parallelism was demonstrated between retraction rate and the platelet content of the blood, even to the extent that thrombocytosis was associated with a slightly hypernormal retraction rate. The relation held, however, only in animals free from infections, for, as in Text-fig. 8, depression in rate took place under conditions of disease without regard for the platelet reading. The extent of retraction was interfered with only when the platelets reached an exceedingly low level.

II. Tumor.

The tumor which forms the basis of these experiments is an epithelioma of variable malignancy (6, 7). It arose spontaneously in a rabbit and has been carried in this species for purposes of study by successive transplantation. Testicular inoculation of tumor tissue, as routine, gives rise invariably to local neoplastic growth. Metastases develop in a high proportion of instances, often very profuse and resulting fatally within a period of from 20 to 80 days. In others, after a certain amount of growth, the neoplasm becomes necrotic and is absorbed, with survival of the animal.

³ Obtainable from Arthur H. Thomas Co., Philadelphia, and from Firma Arno Haak, Jena, Germany.

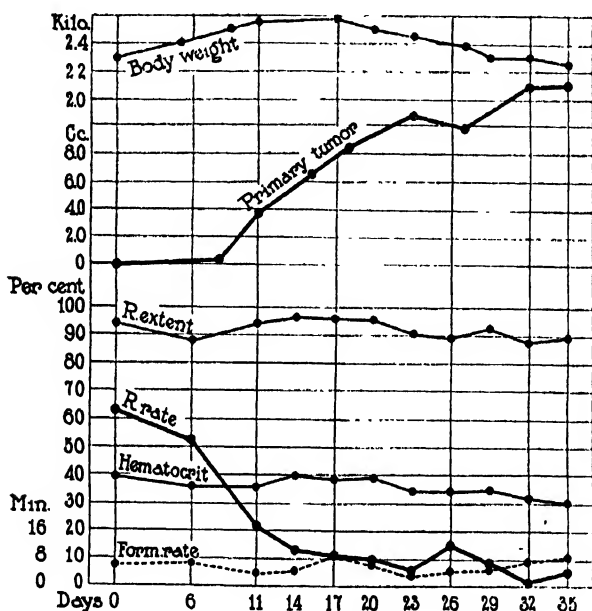
Examination of the blood coagulability has been carried out in 58 rabbits inoculated with tumor, tests being made at regular intervals throughout the course of the disease. In many cases the results have been difficult to interpret because of complication of the picture by the effects of spontaneous infections, most frequently appearing where the tumor ran a long and severe course and resulted in physical depletion of the animal. However, uncomplicated cases are abundantly at hand, and typical examples will be described.



TEXT-FIG. 9.

A very malignant form of the tumor is illustrated by an animal whose chart is given in Text-fig. 9. The primary tumor grew rapidly and on the 18th day a metastasis was discerned in the right iris. This developed vigorously and 3 days later the left eye was similarly involved. During this period the rabbit lost in strength and ate little. The hind quarters were found paralyzed on the 24th day and the experiment was terminated. At postmortem examination the general state of nourishment was found reduced but not depleted. The primary tumor measured 1.5 by 1.5 by 5.0 cm., had completely destroyed the testicle and invaded the spermatic cord. Extensive involvement by metastases of the abdominal viscera was presented, also, of both eyes, lymph nodes of the neck, femoral bone marrow on both sides and spinal column. The blood readings obtained are shown in the chart, a curve being added to represent the rate of growth of the primary

tumor as estimated by measurements during life (8). Shortly after inoculation, at a time when the initial tumor could be felt in the testicle as a pea-sized nodule, a distinct depression was present in clot retraction rate, and this value continued to fall still farther. On the 18th day the reading tended to return somewhat toward normal but soon fell again and reached a very low level at the end of the experiment. The curve of retraction rate, thus, is seen to represent practically an inversion of the growth curve of the tumor. Retraction extent remained within the normal range throughout. Clot initiation and formation were normal until the last 2 days when marked delay occurred in these functions.



TEXT-FIG. 10.

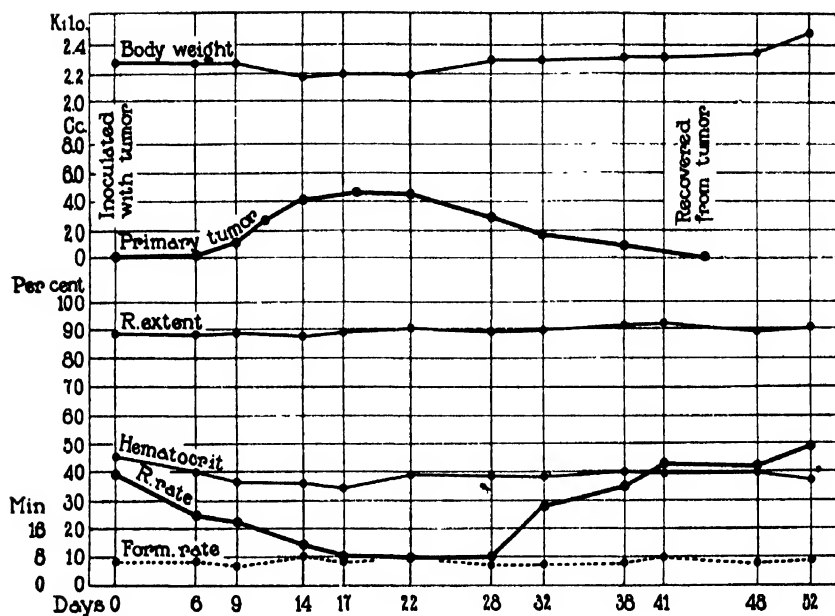
An instance of more gradually extending but severe neoplastic disease is given in Text-fig. 10. The primary tumor developed steadily to large size. On the 19th day the first metastasis was discovered and others followed. From this time on the animal lost in weight and by the 35th day had become emaciated and weak, when it was killed and autopsied. The body tissues were wasted. The primary tumor completely distended the scrotal sac, and tumor nodules were found in the spermatic cord and retroperitoneal lymphatics. The adrenals were largely replaced by metastases; the omentum contained clusters of them. Nodules were present also in the mediastinum, posterior lymphatics of the neck and right iris, and the nasal cavities and sinuses were filled with neoplastic growth. A nodule had entirely destroyed the hypophysis, eroding the sella turcica. In the chart it will be seen that clot retraction rate began to diminish at an early stage of tumor

development and dropped progressively with its further extension, to a low value at death. Here again, the curve of this function parallels inversely the tumor curve. Fluctuations which occurred in the extent of retraction and in clot formation rate were minor and lay within the limits of normal.

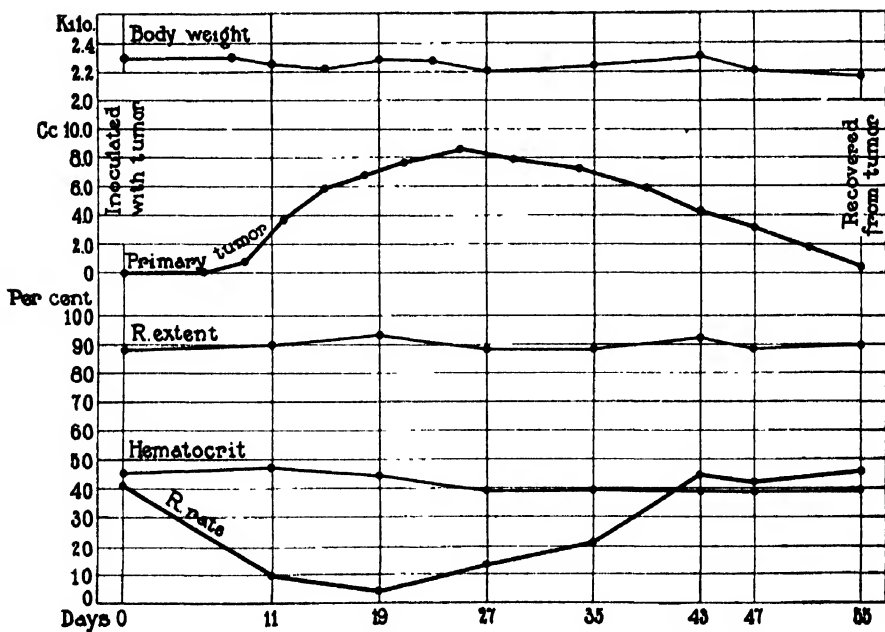
A comparatively benign course of the disease is illustrated in the cases of Text-figs. 11 and 12. The primary tumors grew to moderate (Text-fig. 11) or large (Text-fig. 12) size, and then receded without giving rise to discoverable metastases. The charts show a progressive depression of clot retraction rate during the early stages of the disease, and a return to normal as the tumors become absorbed. Retraction extent and formation rate showed no definite changes.

The rabbits whose charts are given in Text-figs. 13 and 14 are presented as types with complications. In the first, the testicular tumor developed to moderate proportions and metastases were felt in the abdominal wall on the 19th day. The iris of both eyes became involved. After the 25th day the tumors ceased to grow and were eventually absorbed. 48 days after inoculation an attack of snuffles set in with sneezing and profuse nasal discharge. A few days later the experiment was terminated. At postmortem examination the nasal cavities were found filled with pus; other than this and the scar of the healed primary tumor no lesions were present. The chart (Text-fig. 13) indicates that during the period of tumor activity retraction rate was depressed, and showed a secondary depression while the necrotic tumor tissue was being absorbed. After returning finally to a normal level, it dropped a third time at the onset of upper respiratory infection. Other factors in the blood tests were normal throughout.

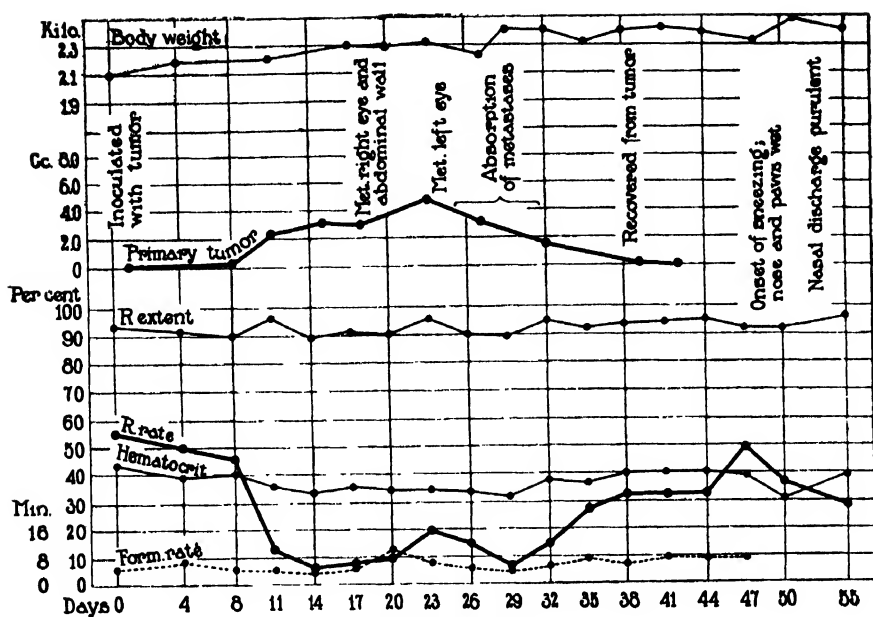
The rabbit of Text-fig. 14 pursued an extended and rather severe form of the disease. The testicle containing the primary tumor was withdrawn into the abdomen soon after inoculation and the growth of the tumor could not be followed. On the 28th day appeared a metastasis in the right iris and others followed in great numbers,—with masses of tumor to be felt in the deep tissues of the neck and in the abdomen. Growths developed at the gingival margins of the upper molar teeth, protruding into the mouth to such an extent as to interfere with eating. From the 30th day on the rabbit became thinner and ultimately cachectic, although it remained vigorous at all times. The experiment was terminated 58 days after inoculation. Autopsy: The tissues were everywhere wasted. An enormous primary tumor lay within the peritoneal cavity, and massive collections of metastases involved the retroperitoneal lymphatics, adrenals, kidneys, spleen, lungs, mediastinum, thymus, neck muscles and lymphatics, hypophysis, both eyes and the maxillary antrum, with infiltration of the alveolar processes and gums. All tumors, however, were thickly encapsulated and entirely necrotic. Evidently the neoplastic process had been arrested for some time, and the cachexia was the combined result of absorption of necrotic tumor tissue and the inability of the animal to take food properly. In the chart, retraction rate is indicated by a 1 hour serum reading in comparison with the final, and this value is seen to have fallen slightly during the period of metastasis (30th to 43rd days), and to have risen to a



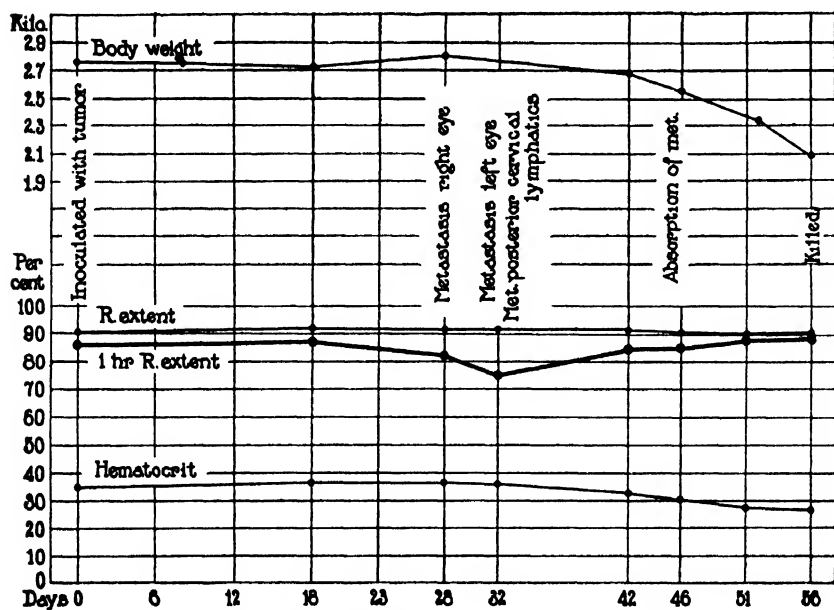
TEXT-FIG. 11.



TEXT-FIG. 12.



TEXT-FIG. 13.



TEXT-FIG. 14.

high position the last week of the experiment. Other factors in the tests showed no alterations of note.

DISCUSSION.

The reports in the literature are very inconsistent with regard to blood coagulability in disease in general, excluding the hemorrhagic diatheses, but where authors have used carefully controlled methods of testing the results indicate that coagulation rate and the retraction of the clot as observed qualitatively remain quite normal. Addis (9), in examining 112 patients, all of whom were seriously ill and presenting various sorts of disease both bacterial and non-bacterial in nature, found changes in coagulation rate in 30 per cent, and these were in an extremely critical condition when the blood was tested. Lee and White (10) conducting a similar investigation also obtained alterations only in the critically ill, and then not constantly. Pneumonia is an exception, for Dochez (11) and others have demonstrated consistent delay in clotting rate during its progressive stages; this is probably to be accounted for in the presence of the pneumococcus toxin (Avery) and its specific action upon elements of the blood connected with coagulation. Imperfectly retracting or non-retractile blood clot has been reported to occur occasionally in certain infections, *i.e.*, diphtheria, pneumonia, miliary tuberculosis, nephritis, ulcerative colitis, smallpox, etc., and these, too, have been cases of unusual severity. Thrombocytopenic purpura hæmorrhagica is the only condition which presents non-retractile blood clot with any regularity. The results of the present work in diseases of the rabbit agree with these views, *i.e.*, alterations in the rate of clot formation and extent of clot retraction were found confined to rapidly progressing pathological conditions, at the height of the disease and at terminal stages, and here inconstantly. They failed entirely to appear in gradually developing maladies, even where profound emaciation and weakness were reached.

One factor of the blood coagulative process, however, the rate with which the clot retracts, was found very sensitive to the presence of disease whether local or general in nature. Lesions without apparent constitutional effects, a small blistered burn, a sterile incision, a tumor of pin-head size, produced distinct fluctuations of this value. The

onset of rhinitis was indicated before the appearance of symptoms. The relation of clot retraction rate to the course of disease could be traced with particular assurity in the case of the tumor where a measure was also afforded of the progress of the lesion. Here, not only was the curve of retraction rate, in uncomplicated instances, nearly an exact reflection of the curve of tumor growth, but metastases were detected during or immediately following periods in the disease characterized by very low retraction rate readings. That reduction in the rate of clot retraction has to do with disease activity rather than with a poor state of bodily nutrition *per se*, is demonstrated by the fact that body weight was found to run parallel to retraction rate only in so far as it also reflected the stress of disease. Examples from the cases cited illustrate this: While in Text-fig. 11 body weight and clot retraction rate were simultaneously reduced during the active stages of tumor growth, in Text-figs. 9 and 10 the animals gained steadily in weight with the first inroads of the tumor, and retraction rate fell; in Text-fig. 14, when disease activity had totally ceased and cachexia was extreme, the rate of retraction remained at a normal level. There is also no consistent correspondence between anemia (hematocrit values) and defective clot retraction.

The alterations in blood composition that are responsible for changes in clot retraction have not been entirely explained. Hayem (12) showed that blood plasma deprived of all cellular elements formed a firm clot on standing and that the clot failed to retract. On the other hand, when he allowed the platelets alone to remain in the plasma, the clot which formed retracted normally. It was pointed out by Duke (13) that in purpura hæmorrhagica with a reduction in the number of platelets in the blood below about 50,000 per c.mm., specimens of blood formed non-retractile clots, and le Sourd and Pagniez (14), among others, have been able to induce clot retraction in such blood specimens by adding normal platelets. However, the latter authors (15) came to the conclusion, after both clinical and experimental observation, that, while the presence of blood platelets seems indispensable for normal retractility of the blood clot, retraction occasionally fails to take place even when platelets are present in abundance. This has been our experience, also, with regard to the relationship of both rate and extent of clot retraction to the blood

platelet content. It is possible also that the quality (fragility or chemical nature) of the platelets, as well as the quantity, may vary and play a rôle in clot retraction. The fact is well known, moreover, that the addition to the blood of minute traces of acid changes markedly the consistency of the clot, and the hydrogen ion concentration of the blood may readily be a determining factor in clot retractility.

SUMMARY.

Description is given of changes in blood coagulability found in diseases of the rabbit, including malignant tumor, spontaneous infections, non-bacterial diseases and lesions, and hemorrhagic states specifically induced. The changes involved variously the time of onset of blood coagulation, clot formation rate and the rate and extent of clot retraction.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROID-ECTOMIZED RABBITS.

I. THE EFFECTS OF PARTIAL PARATHYROIDECTOMY.

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Observations on the endocrine tissues of rabbits irradiated with a quartz mercury arc lamp have disclosed the striking hypertrophy of the parathyroid glands produced by the irradiation (1). Histologically, this hypertrophy was found to be a true hyperplasia of the endocrine tissue, but a study of the calcium and inorganic phosphate content of the blood of these rabbits failed to reveal any significant changes from the normal levels (2).

That parathyroid integrity is essential to the maintenance of a normal blood calcium level has been established by the extirpation experiments of many investigators, but the upper limit of calcium concentration seems to be determined by factors independent of parathyroid control. Hence, in the normal animal, parathyroid hypertrophy due to an external stimulus, such as light, appears to result only in an increase in the factor of safety, and to be unaccompanied by a recognizable increase in functional activity for which there is no physiological demand.

On the other hand, the presence of an increased factor of safety might be revealed by an unusual functional strain or emergency, and a study of such conditions, experimentally produced, might throw further light upon normal parathyroid function. Having at hand methods of producing a hypertrophic condition of the parathyroid glands by means of ultra-violet light, and a condition of reduced function by partial extirpation, we undertook a series of experimental comparisons of groups of rabbits with enlarged, normal, or partially extirpated parathyroids.

In preparation for these studies, partial parathyroidectomies were performed on a number of rabbits, some normal, some after irradiation with ultra-violet light. The effects of the operation itself upon the calcium and the inorganic phosphate level of the blood of these animals form the subject matter of this paper.

Experimental Procedure.

Since the partially parathyroidectomized rabbits were to be used subsequently in other experiments, the operations were performed on small groups of animals and at various seasons of the year. In some instances only the two external parathyroids were removed; in others, the thyroid gland was divided at the isthmus, and one lobe, with its internal parathyroid gland, was removed also. The animals were all healthy, adult, albino males, and the glands were removed aseptically, under ether anesthesia, after a median incision and blunt dissection of the tissues of the neck. The operative wounds healed without infection. At intervals after the operation, small samples of blood were taken from the marginal ear vein and allowed to clot, and calcium (3) and inorganic phosphorus (4) determinations were made on the serum.

Preliminary observations on two normal rabbits, after removal of the two external parathyroid glands, showed that the operation was followed by a sharp drop in blood calcium to a low level on the 2nd or 3rd day, and a more gradual increase toward the normal over a period of days or even weeks. No signs of tetany appeared in these two animals.

Experiment 1.—The next groups of six and four normal rabbits respectively were deprived of both external parathyroid glands and one internal gland also. In spite of individual variations in reaction, the trends of the blood calcium curves of these rabbits were essentially similar. Immediately after operation, the blood calcium of all ten rabbits dropped from normal levels, between 11.6 and 13.0 mg. per 100 cc. of serum, to 6.2 to 10.2 mg. (37 to 88 per cent of the control figures) by the 2nd day. The rabbit with 6.2 mg. of calcium per 100 cc. of blood serum succumbed on that day in severe tetany. From this point the blood calcium of seven of the remaining nine rabbits began to rise towards normal, so that figures of 10.8 mg. to 12.6 mg. were obtained on the 4th to the 6th days, and these levels were usually maintained thereafter (Chart 1). The other two rabbits had persistently lower blood calcium levels, and one of them showed a drop from 8.5 mg. per 100 cc. of serum on the 14th day after operation, to 4.8 mg. on the 17th day, when it had a typical tetanic seizure and died. The other rabbit maintained a serum calcium level between 8 and 10 mg. for 35 days. 2 days later it was transferred to a fasting

experiment, in which it succumbed on the 5th day. On the day before death its blood calcium was 5.65 mg. per 100 cc. of serum.

The inorganic phosphorus in the blood serum of most of these rabbits was not examined at regular intervals. It was observed, however, that before death the serum phosphorus of the rabbits that succumbed on the 2nd, 17th, and 42nd days after operation was 9.4 mg., 13.1 mg., and 12.0 mg. respectively per 100 cc. of serum, as compared with a normal average of 7.4 mg.

In this experiment the capacity of the residual parathyroid tissue to respond to sudden demands is shown by the fact that in seven of ten rabbits, after the initial drop, blood calcium levels only 5 to 10 per cent below normal were attained within a week, and these levels were usually maintained thereafter. If, after partial para-

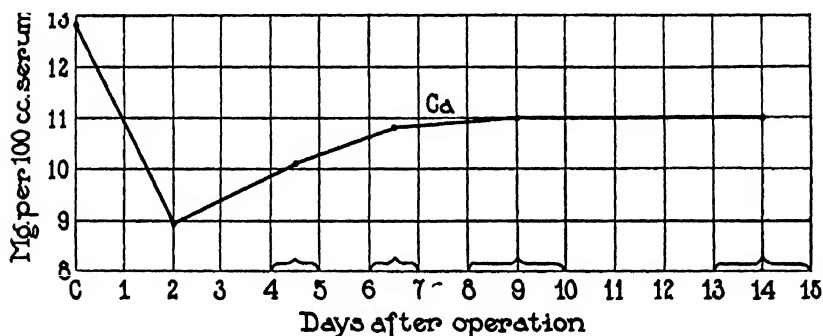


CHART 1. Experiment 1. Serum Ca levels after partial parathyroidectomy.

thyroidectomy in normal rabbits, the remaining tissue is usually able to restore the blood calcium level nearly to normal in 4 to 6 days, this is the interval in which evidence of parathyroid hyperplasia and consequent increase in the factor of safety must be sought in irradiated rabbits.

In the following experiment, normal and irradiated rabbits were partially parathyroidectomized and observed during the ensuing period.

Experiment 2.—A group of ten adult albino rabbits had both external parathyroids removed. Five of these animals, with backs clipped and ears shaved, had been exposed for 30 minutes a day, 6 days a week, for 3 weeks, at 1 meter distance, to a quartz mercury arc lamp (67 volts, 5.5 amperes). These rabbits presumably had hypertrophic parathyroid glands (1). The exposures were continued for the further period of observation. The five other rabbits served as controls for the

irradiated group. All were bled for a serum sample before operation, on the 1st, 2nd, and 4th days following, and then every 2 or 3 days to the 15th or 16th day.

A difference in the reaction of the irradiated and control rabbits to the loss of their external parathyroids became apparent within 24 hours after operation. None of the irradiated rabbits showed signs of tetany, but three of the controls were observed in acute attacks, characterized by gross and fibrillar twitchings of the voluntary muscles,—notably those of the jaw,—tonic and clonic convulsions, opisthotonos, deep, heaving respiration, venous engorgement, and

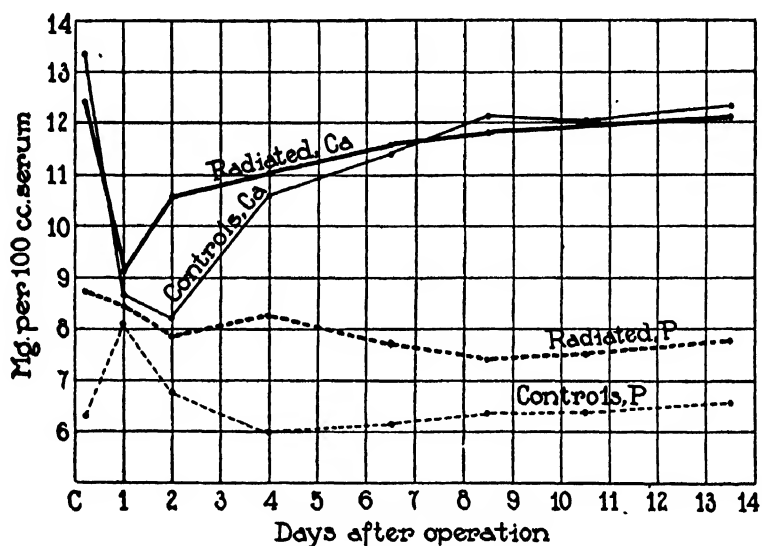


CHART 2. Experiment 2. Comparison of serum Ca and P in irradiated and control rabbits after partial parathyroidectomy.

excessive salivation. In two of these rabbits an intravenous injection of 10 cc. of $\frac{N}{5}$ CaCl_2 relieved the signs immediately, but one of them succumbed during the following night. The third rabbit recovered spontaneously.

The absence of all signs of tetany in the five irradiated rabbits led us to infer that the hyperplasia of the internal glands produced by the radiations had increased their functional capacity, *i.e.*, their factor of safety, to a degree sufficient to protect the animals. This increase in the factor of safety in the irradiated rabbits is reflected in a comparison of their blood calcium and inorganic phosphorus

curves. In both groups the fall in calcium was immediate, so that 24 hours after operation the average for the irradiated rabbits had dropped from 12.4 to 9.1 mg. (73 per cent of normal), and for the controls from 13.4 to 8.7 mg. (65 per cent of normal) per 100 cc. of serum. But the following day the blood calcium average in the irradiated group rose to 10.6 mg. per 100 cc. of serum, while the four survivors of the control group suffered a further loss to 8.2 mg. By the 7th day this difference had been overcome, and both groups then maintained calcium levels approaching normal (Chart 2). Similarly, due to sharp rises in the blood phosphorus of the three control rabbits

TABLE I.

Blood Calcium and Inorganic Phosphorus 1 Day after Partial Parathyroidectomy.

Normal rabbits				Irradiated rabbits			
No.	Calcium	Phosphorus	Ratio $\frac{\text{Ca}}{\text{P}}$	No.	Calcium	Phosphorus	Ratio $\frac{\text{Ca}}{\text{P}}$
	Mg. per 100 cc. serum				Mg. per 100 cc. serum		
18	10.9	6.0	1.82	13	10.3	8.5	1.21
19	9.5	4.8	1.96	14	10.1	7.2	1.40
20*	8.5	8.7	.98	15	8.9	8.6	1.03
21†	8.0	11.0	.73	16	8.5	8.7	.98
22*	6.0	9.9	.61	17	7.8	9.2	.85

* Observed in tetany.

† Died.

that had outspoken tetany, the average for the group was carried upward on the 1st day from 6.3 mg. to 8.1 mg. per 100 cc. of serum. Meanwhile, in the irradiated group, with a high average blood phosphorus to start with (2), only one of the rabbits showed a further rise in blood phosphorus following the operation. This rabbit had the greatest drop in calcium of any in the irradiated group.

Such a reciprocal relationship between blood calcium and phosphorus, pointed out by Howland and Kramer (5), and recently observed in normal dogs by Mayerson, Gunther, and Laurens (6), is illustrated in this experiment by arranging the rabbits of each group in the order of their blood calcium levels on the day following partial parathyroidectomy (Table I). In both groups the rabbits

with the lower calcium values after partial parathyroidectomy had the higher figures for blood phosphorus.

A final group of five normal rabbits, whose external parathyroid glands were extirpated, confirmed the earlier observations by exhibiting an average fall of blood calcium from 12.0 mg. to 8.8 mg. per 100 cc. of serum, 24 hours after operation. In three of these rabbits the calcium level was restored to normal in 7 to 9 days. The other two had persistently lowered blood calcium, and one rabbit, after transfer to a fasting experiment, on the 9th day, suffered a further drop in Ca to 4.9 mg. and died on the 16th day after the partial parathyroidectomy. 2 days before death this rabbit's blood

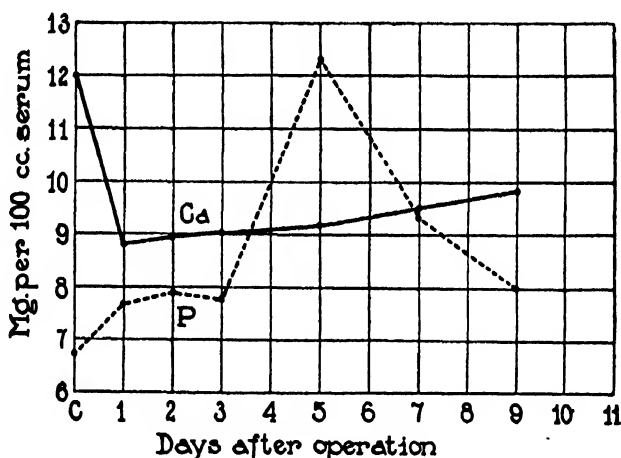


CHART 3. Serum Ca and P levels after partial parathyroidectomy.

phosphorus was 12.68 mg. per 100 cc. of serum. All the rabbits in this group exhibited a high blood phosphorus at one time or another after operation, the average for the group on the 5th day being 12.3 mg. per 100 cc. of serum, compared with a control level before operation of 6.7 mg. (Chart 3).

That the ether anesthesia and the operative procedure did not contribute to the fall in serum calcium, or the rise of inorganic phosphorus, was shown by the absence of such changes in two control rabbits, in which identical operations were performed, except for the removal of the parathyroid glands.

The Relative Significance of a Fall in Blood Calcium and of a Rise in Blood Phosphorus in Parathyroid Tetany.

We have already noted the marked rise in the inorganic phosphorus of the blood serum, which often accompanied the drop in serum calcium after partial parathyroidectomy. A rise in inorganic phosphorus occurred in some rabbits which did not develop tetany, but it was never absent in those that did, and was apparently associated with the onset of the acute attack. The question arises whether it is only the fall in calcium, or in reality the accompanying change in the ratio of calcium to phosphorus in the blood serum that is more significant in tetany.

TABLE II.

Rabbit No.	Onset of tetany day after operation	Ca	P	Ratio $\frac{\text{Ca}}{\text{P}}$	Outcome
		Mg. per 100 cc. serum			
4	17th	4.8	13.1	.37	Died
6	41st	5.7	12.0	.48	Died
9	2nd	6.2	9.4	.66	Died
21	1st	7.9	11.0	.72	Given CaCl_2 ; died
20	1st	8.5	8.7	.98	Mild attack; recovered
22	1st	6.0	9.9	.61	Given CaCl_2 ; recovered
26	16th	4.9	12.7	.38	Died
Average.....		6.3	11.0	.60	

Normally, in rabbits, this ratio $\frac{\text{Ca}}{\text{P}}$ is not less than 1, and it usually lies between 1.3 and 2.6. Among 103 normal rabbits, with a calcium level, on admission, between 11.1 and 13.9 mg. per 100 cc. of serum, the lowest ratio $\frac{\text{Ca}}{\text{P}}$ was 1.10 and the three highest, with one exception, were 3.3. The exceptional rabbit had a very low serum phosphorus (1.8 mg.) and a unique ratio $\frac{\text{Ca}}{\text{P}}$ of 7.30. The average ratio for all the rabbits was $\frac{12.4}{6.4} = 1.9$.

In the rabbits that developed acute tetany after partial parathy-

roidectomy, on the other hand, the ratio $\frac{\text{Ca}}{\text{P}}$ was changed not only by the fall in calcium, but by an abnormal rise in phosphorus also. Table II shows the ratio $\frac{\text{Ca}}{\text{P}}$ in these rabbits, according to the last figures obtained before the attack, and it will be noted that high phosphorus figures contribute as much to change the $\frac{\text{Ca}}{\text{P}}$ ratio from normal, as do the low figures for blood calcium.

Of nine other partially parathyroidectomized rabbits, which were not observed in tetany, four at one time or another had calcium

TABLE III.

Rabbit No.	No. of days after operation	Lowest calcium level	Phosphorus level	Ratio $\frac{\text{Ca}}{\text{P}}$
		Mg. per 100 cc. serum		
3	2	8.6	7.7	1.12
5	2	7.6	8.6	.88
7	18	10.6	5.7	1.9
19	2	8.4	5.6	1.5
18	1	10.9	6.0	1.82
8	1	9.2	8.0	1.15
10	1	9.6	10.8	.89
11	5	7.3	12.2	.60
12	3	6.6	8.1	.81

and phosphorus levels comparable with some of those in the tetanic animals. The other five did not have either very low calcium or high phosphorus at any time after operation, and their $\frac{\text{Ca}}{\text{P}}$ ratios were usually more than 1 (Table III).

DISCUSSION.

Our observations are in accord with those of other investigators who have studied calcium and phosphorus metabolism after parathyroidectomy. The characteristic drop in blood calcium has often been found and needs no further comment. This fall in blood calcium is certainly a primary reaction, which may or may not be followed by a

rise in phosphorus, either immediately, or later, when the blood calcium is returning toward the normal level.

A phosphorus retention, following parathyroidectomy in dogs, was observed in 1911 by Greenwald (7), who has since published a series of papers in which the work of others is reviewed. He has recently stated (8, *a*) that in over 50 parathyroidectomized dogs tetany never appeared without phosphorus retention, and conversely, a drop in phosphorus excretion after attempted parathyroidectomy was observed in only one of ten dogs that did not develop tetany. However, the increase in "acid-soluble" phosphorus in the blood of parathyroidectomized dogs in tetany, which Greenwald had observed in 1913 (8, *b*), was not so marked in his later experiments, and in 1924 (8, *c*) he appears to minimize its significance, assuming that the excess of phosphates retained is deposited in the tissues, and only reappears in the circulation under certain abnormal conditions, such as after ether anesthesia, or cerebral or general anemia. In those of our rabbits that developed tetany, an excess of phosphorus was found in the blood.

Salvesen (9) also found that the inorganic phosphorus of the blood serum of completely parathyroidectomized dogs was considerably increased, and tended to remain above the normal value. According to one protocol, a dog with a normal blood calcium of 10 mg. per 100 cc. of serum and inorganic phosphorus of 3.0 mg. ($\frac{\text{Ca}}{\text{P}} = 3.3$) developed severe tetany 2 days after complete parathyroidectomy, with a $\frac{\text{Ca}}{\text{P}}$ ratio of $\frac{3.50}{12.5} = 0.28$, and the serum phosphorus level remained above 6.0 mg. per 100 cc. of serum thereafter.

In a study on blood changes and clinical symptoms, following oral administration of phosphates in dogs, Salvesen, Hastings, and McIntosh (10) discuss the changes in blood phosphorus and calcium that result, and they conclude that the fall in calcium is the significant factor in the development of tetany.

In a recent private communication, Dr. John Howland stated his conviction that the clinical syndrome known as infantile tetany is certainly not the result of a high phosphorus content of the serum which drives down the calcium concentration (11), and observed that infantile tetany is found at times with a very low phosphorus concentration of serum. He has found that in many instances the phosphorus is high, perhaps in two-thirds of the cases, but there is the one-third which cannot be neglected.

We shall postpone further discussion of the subject until we report a series of observations on rabbits to which secondary sodium orthophosphate was given subcutaneously. The point to be noted at this time is that the calcium level itself was apparently not the sole determining factor in the development of tetanic signs in these partially

parathyroidectomized rabbits. Only in connection with an increase in blood phosphorus, which further disturbed the normal $\frac{\text{Ca}}{\text{P}}$ ratio, were signs of hyperirritability observed.

SUMMARY.

Previous irradiation of rabbits with ultra-violet light, with a consequent hypertrophy of the parathyroid glands, resulted in some degree of protection to these animals, when part of the parathyroid tissue was extirpated. This fact indicates that the remaining hyperplastic tissue was potentially active, and that the increased factor of safety represented by this condition of the remaining tissue resulted in less loss of blood calcium, a more rapid initiation of recovery, and an absence of the rise in the inorganic phosphorus of the blood serum which was a characteristic result of partial parathyroidectomy in normal rabbits. A fall in calcium in all the irradiated rabbits, without a corresponding rise in phosphorus, shows that the drop in calcium is the primary reaction, and a rise in phosphorus a secondary reaction following parathyroidectomy. But the relation of the rise in phosphorus to the development of tetany remains obscure.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROID-ECTOMIZED RABBITS.

II. THE EFFECTS OF INJECTIONS OF CALCIUM CHLORIDE OR OF DISODIUM HYDROGEN PHOSPHATE.

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(Received for publication, September 11, 1926.)

Although the calcium and inorganic phosphorus levels of the blood serum of rabbits appear to be more affected by seasonal changes in environment and by caging (1) than by the hypertrophy of the parathyroid glands induced by ultra-violet radiations (2), the hypertrophic changes in the glands are evidence of an increased "factor of safety" which is manifest in the reactions of irradiated animals to partial parathyroidectomy (3). A comparison of their behavior with that of normal rabbits subjected to an identical operation¹ led us to a further investigation of the reactions of irradiated, normal, and partially parathyroidectomized rabbits to other experimental procedures, in order to test the functional capacity of their parathyroids. The three groups of rabbits were assumed to differ in the relative amount and potential activity of their parathyroid tissue, the irradiated animals having hypertrophic glands and the partially parathyroidectomized rabbits having only so much parathyroid tissue as was necessary for the maintenance of a blood calcium level approaching normal.

Our first observations on the effects of the injection of calcium chloride or of disodium hydrogen phosphate into irradiated, normal, and partially parathyroidectomized rabbits gave some unexpected results, and turned our immediate attention from the primary object of the investigation to an interest in the rôle of inorganic phos-

¹All operations for removal of parathyroids were performed under full ether anesthesia.

phates in the development of outspoken clinical tetany, under conditions of lowered blood calcium.

Calcium Injections.

Two experiments were performed, the first with four rabbits; the second with seven.

Experiment 1.—Two normal, adult, male rabbits served as controls. The other two, with backs and ears closely clipped, had been exposed daily for 30 days to the

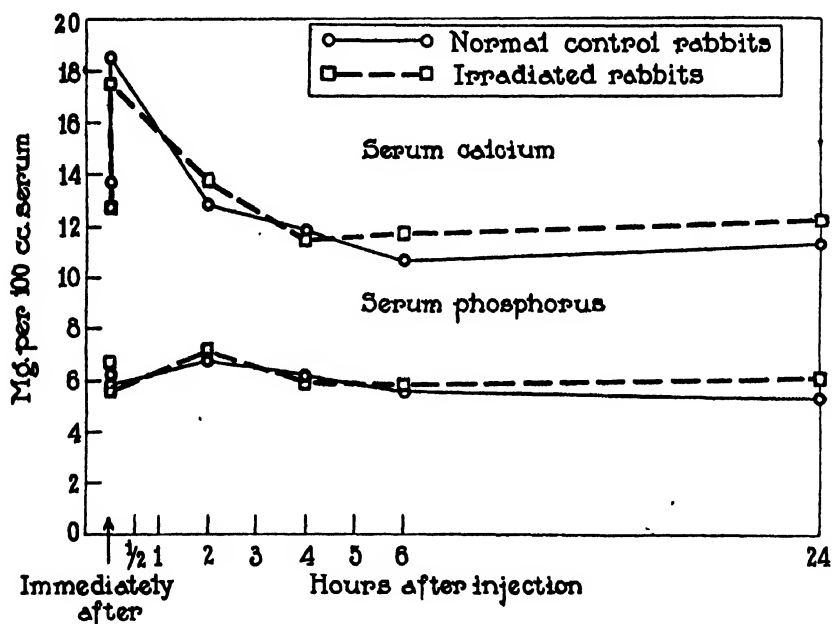


CHART 1. Experiment 1. Serum Ca after injection of CaCl_2 in normal and irradiated rabbits.

total radiations of a quartz mercury arc lamp (67 volts, 5.5 amperes), at 1 meter's distance. During this period the daily exposures were increased from 10 minutes to 2 hours. Then all four rabbits received an intravenous injection of sterile $\frac{M}{8}$ CaCl_2 solution, representing 25 mg. of Ca per kilo body weight. The volume of fluid was about 10 cc. Blood samples were taken before and immediately after the injections, and 2, 4, 6, and 24 hours later, and calcium (4) and inorganic phosphorus (5) determinations were made on the serum.

Chart 1 shows that the reactions of the normal and the irradiated rabbits were essentially similar. The excess calcium practically

disappeared from the blood in less than 2 hours, and the calcium level subsequently fell somewhat below that of the control specimens. This dose of calcium chloride had only a relatively slight effect upon

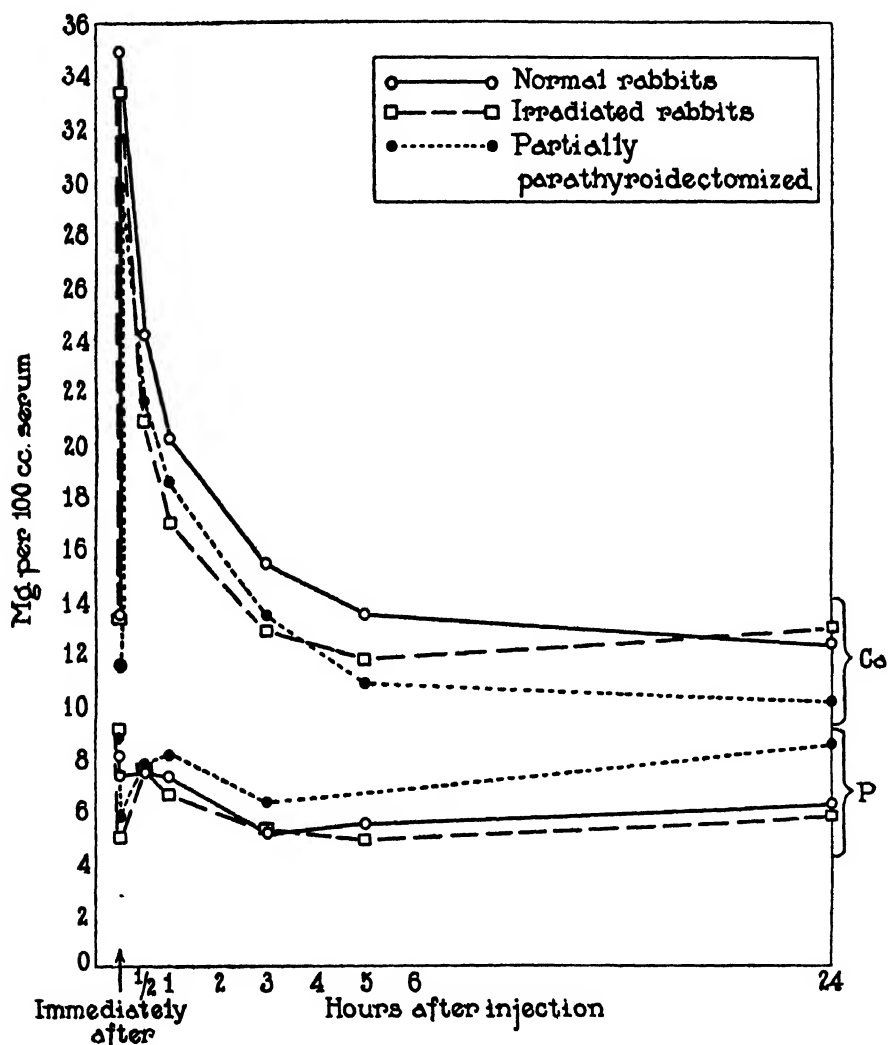


CHART 2. Experiment 2. Serum Ca after injection of CaCl_2 in normal, irradiated, and partially parathyroidectomized rabbits.

the inorganic phosphate levels. The initial drop is due partly to dilution of the blood serum. A reciprocal relationship is seen in the slight rise during the rapid drop in calcium.

Experiment 2.—In this experiment two normal and two irradiated rabbits were supplemented with three partially parathyroidectomized animals. Two of these had been deprived, 6 weeks before, of both external parathyroids and one-half the thyroid with its internal parathyroid gland. The other rabbit had had its external parathyroids removed 4 weeks previously. During these intervals the serum calcium of these rabbits had been gradually restored to 11.4, 12.0, and 11.2 mg. respectively, indicating a corresponding restoration of parathyroid function.

These rabbits received intravenous injections of CaCl_2 in sterile $\frac{M}{4}$ solution, in doses of 35 to 50 mg. of Ca per kilo of body weight. Because of the increased concentration, the total volume injected was somewhat less than in Experiment 1. Small blood samples were withdrawn from an ear vein, just before and after injection, and 30 minutes, 1, 3, 5, and 24 hours later. The results of Ca and P determinations on these specimens are shown in Chart 2.

In all of these rabbits the immediate rise of the blood Ca to a high level was followed by a rapid disappearance of the excess from the blood, so that the control levels were restored in 3 to 5 hours. No striking differences were observable between the average values for the three groups, and indeed the individual figures obtained 30 minutes and 1 hour after injection were remarkably similar throughout. Apparently the condition of the parathyroid glands had little influence on the disappearance of excess calcium from the blood stream. It is interesting to observe in the partially parathyroidectomized rabbits that the blood calcium returned to its former lower level, as Salvesen (6) and others have observed repeatedly under similar conditions.

Thus the calcium level of the blood seems to be determined by a balance between parathyroid activity and other factors independent of parathyroid control. When there is a reduction of parathyroid activity, as after partial parathyroidectomy, the calcium level drops correspondingly. But even an excess of parathyroid tissue does not maintain an excess of calcium already present in the blood, in opposition to the forces which tend to restore it to a normal level (2).

That the immediate reduction of inorganic phosphorus was due in part to the calcium content of the injection fluid is shown by the greater fall in phosphorus in Experiment 2, as compared with Experiment 1, in which a larger volume of fluid was injected. This drop was most marked in the irradiated rabbits of both series, in which an average fall from 7.83 mg. to 5.32 mg. per 100 cc. of serum occurred. The fall in phosphorus was as temporary as the cor-

responding rise in calcium and illustrates again the tendency to reciprocal reactions which several authors have already pointed out. The experiment does not indicate whether the restoration of inorganic phosphorus in the first half-hour after the calcium injection was a cause or an effect of the correspondingly rapid fall in calcium.

Phosphate Injections.

Subcutaneous injections of sterile disodium hydrogen phosphate (Na_2HPO_4) were given in $\frac{\text{M}}{2}$ solution in doses of 100, 125, or 150 mg. of P per kilo body weight.

Experiment 3.—The ten rabbits injected with 100 mg. P per kilo reacted in an essentially similar manner. Five were normal, three had been partially parathyroidectomized (two external glands and one-half the thyroid extirpated 16 to 28 days previously), and two had been irradiated daily for 23 or 26 days in increasing periods of 10 to 90 minutes at 1 meter's distance from the quartz mercury arc lamp. Blood samples withdrawn from an ear vein before the injection, and 4, 6, 12, and 24 hours afterwards were allowed to clot and the sera were analyzed for Ca (4) and inorganic P (5).

The average figures for each group closely represent the individual findings. These averages, plotted in Chart 3, show, in the first place, the somewhat lower blood calcium of the partially parathyroidectomized rabbits before the injection, and a relatively high blood phosphorus in both irradiated (3) and partially parathyroidectomized animals (7). No significant differences occurred in the reactions of the normal and the irradiated rabbits, and the partially parathyroidectomized animals differed from the others only in the fact that the lower calcium level which distinguished them was maintained throughout the period of observation. The subcutaneous injection of disodium hydrogen phosphate was followed by a prompt rise of inorganic phosphorus in the blood. The peak of the rise, as the next experiment will show, probably occurred before the 4 hour blood sample was taken. In all of these rabbits the excess phosphorus in the blood was practically eliminated within 12 hours. In fact, the most striking feature of the curves is the drop in the blood phosphorus of the irradiated and the partially parathyroidectomized rabbits well below the high levels found in their blood before the injection.

Since the blood is normally a supersaturated solution of calcium bicarbonate and calcium hydrogen phosphate (8), it is to be ex-

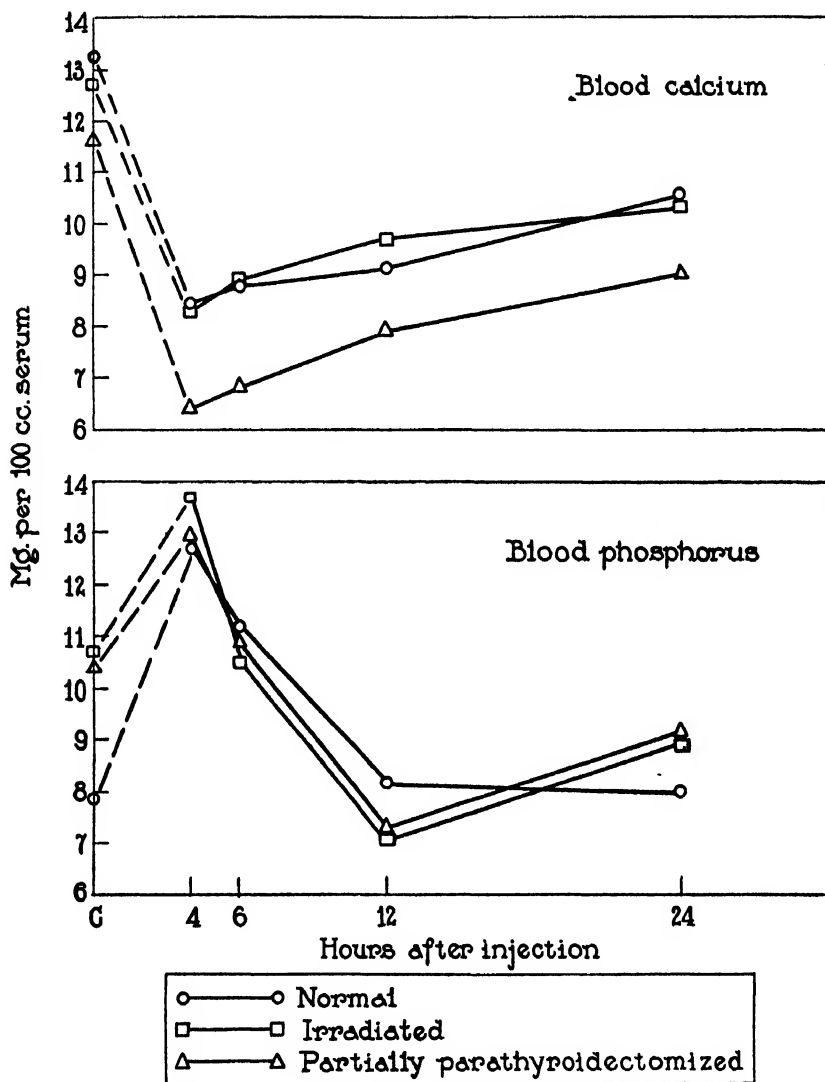


CHART 3. Experiment 3. Serum Ca and P after injection of Na_2HPO_4 in normal, irradiated, and partially parathyroidectomized rabbits.

pected that a rise in other phosphates would cause a fall in calcium. Binger (9) produced tetany in dogs by phosphate injections and found the blood calcium reduced beyond the degree attributable

to serum dilutions. Salvesen, Hastings, and McIntosh (10) have recently raised the blood phosphorus level in dogs by oral administration of mono- and disodium orthophosphates, and observed a corresponding fall in blood calcium.

In this experiment the blood calcium level fell promptly in all the rabbits, probably reaching its nadir about the 4th hour, and then rising slowly toward normal. Some scattered observations after 48 and 72 hours indicate that the normal levels were attained within that time. The lowest Ca level recorded was 6.0 mg. per 100 cc. serum, found 4 hours after the phosphate injection in a partially parathyroidectomized rabbit. This rabbit showed no outspoken tetany, but another partially parathyroidectomized animal with a Ca level of 6.4 mg. had a single convulsive seizure 6 hours after injection. All of the rabbits were restless and hypersensitive, but no other acute attacks of tetany were observed.

Although they were studied in small groups, all the rabbits that received subcutaneous injections of $\frac{M}{2}$ disodium hydrogen phosphate, representing 125 or 150 mg. of P per kilo body weight, may be considered together, for the size of the dose does not serve to distinguish their reactions, which depended rather upon the calcium and phosphorus levels attained in the blood.

Experiment 4.—Nineteen rabbits in four groups are included in this experiment: (1) four rabbits were normal, adult males; (2) four rabbits had been irradiated, three of them for 15 to 25 minutes 6 days a week for 26 to 30 days, at a distance of 50 cm. from a quartz mercury arc. The other had been exposed 10 to 90 minutes a day for 26 days at 1 meter's distance; (3) five rabbits had been irradiated with the quartz mercury arc (30 minutes daily, 18 to 21 days, at 1 meter's distance), and then partially parathyroidectomized with the removal of the two external parathyroids only. Irradiation was continued for the 13 to 16 days between the operation and the date of the phosphate injections; (4) six rabbits had not been irradiated, but underwent similar operations 13 to 16 days before the experiment. Disodium hydrogen phosphate in $\frac{M}{2}$ solution was given to all these rabbits subcutaneously in single doses, representing 125 to 150 mg. P per kilo body weight. Blood samples taken before and at intervals after the injections were analyzed for Ca and inorganic P.

In all nineteen rabbits the immediate results of these phosphate injections were a rise in blood phosphorus, a drop in the blood calcium level, and the onset of signs and behavior characteristic of

acute tetany. The reaction usually was most severe between the 4th and 8th hours, an interval in which ten rabbits died. Another

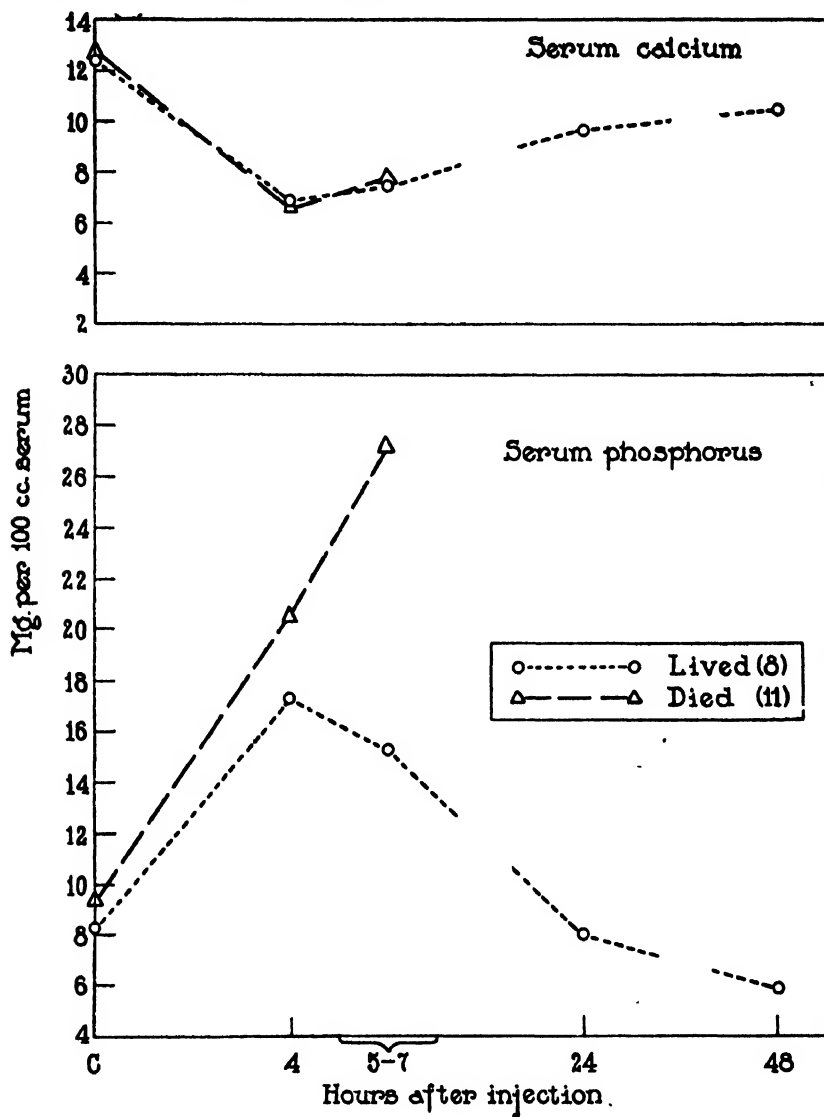


CHART 4. Experiment 4. Serum Ca and P after injection of Na_2HPO_4 showing association of P retention and death.

rabbit was found dead the following morning. The other eight, after exhibiting varying degrees of tetany, survived and returned to a state of normal irritability in 24 to 48 hours.

As already stated, the object of these experiments was to observe possible differences in the behavior of irradiated, normal, and partially parathyroidectomized rabbits to sudden changes in the calcium and phosphate balance in the blood. As in the first three experiments, so in this one a review of the rabbits' reactions shows that they cannot be distinguished according to the apparent state of the animal's parathyroid glands. Equal numbers lived and died in each group, except that among the irradiated, partially parathyroidectomized rabbits one lived and four died. Possibly these animals were more susceptible than those in the other groups. On the whole, the experiment failed to show differences in reaction which might be referred to differences in parathyroid function due to irradiation or partial parathyroidectomy.

But an analysis of the figures shows that a significant division may be made between all those that lived and those that died, on the basis of their blood calcium and phosphate levels at the onset of tetany (Chart 4). We may therefore turn our attention to this aspect of the situation and analyze the experimental findings from this point of view. The rabbits, regardless of the apparent state of their parathyroid glands, have been grouped accordingly in Table I.

In the first place, the control figures for both groups show less variation in the calcium levels than in those for phosphorus, before injection. Those that lived are not different in blood calcium from those that died, either individually or on the average. But in the group of eight survivors there are only three with a blood phosphorus, before injection, above 7 mg. per 100 cc. of serum, while among those that died nine of the eleven had high control levels, indicating, possibly, an inherent tendency to phosphorus retention.

As is shown by the available 2 hour determinations, the first peak of phosphorus in the blood probably occurred in most instances before the 4 hour specimen was taken. Of the nine rabbits examined at 2 hours, four survived and five died. Those that survived showed at the 4 hour period a considerable drop in blood phosphorus to a level which was maintained or further decreased at 5 to 7 hours. Some rabbits that died, on the other hand, had even higher levels at 4 hours than at 2, and the high concentrations of phosphorus in their blood at 5 to 7 hours are in marked contrast to the correspond-

TABLE I.

Lived													
Rabbit No.	Condition	Serum Ca mg. per 100 cc.						Serum P mg. per 100 cc.					
		Control	Hrs. after injection					Control	Hrs. after injection				
			2	4	5-7	24	48		2	4	5-7	24	48
22	N	11.0	6.3	5.7	7.2	8.8	11.2	5.6	19.7	15.6	14.6	7.5	6.5
24	N	12.3	7.2	6.5	6.3	7.8	11.0	5.2	18.1	16.8	17.0	9.0	5.8
26	I	14.6	—	7.4	7.8	10.2	11.0	13.0	—	17.1	17.4	9.6	—
29	I	12.8	8.6	7.9	7.9	12.4	—	6.6	25.7	17.1	11.5	5.0	—
35	PP	12.8	—	7.4	—	6.4	5.8	15.3	—	20.6	—	18.0	7.4
34	PP	13.7	—	6.7	—	11.8	12.7	5.7	—	12.8	—	4.3	5.2
31	PP	12.9	—	7.3	—	10.8	11.4	5.8	—	15.1	—	4.7	4.9
37	IPP	10.8	7.5	6.7	8.1	9.2	—	9.5	29.2	23.8	15.7	6.0	—
Average.....		12.6	—	7.0	7.5	9.7	11.0	8.3	—	17.4	15.2	8.0	6.0
						Died						Death at	
25	N	13.3	7.5	5.5	6.7			10.3	24.2	25.6	22.1	5 hrs., 18 min.	
23	N	11.9	7.5	6.7	7.9			9.9	17.2	19.2	22.5	6.05	
27	I	13.1	7.3	6.3	6.5			12.0	27.7	21.7	21.7	7-8 hrs.	
28	I	12.0	8.6	8.2	8.1			6.2	24.0	18.4	24.8	First night	
30	PP	13.0	—	7.1	7.3			6.8	—	14.8	33.0	7.05	
32	PP	12.9	—	6.5	—			7.6	—	25.9	—	3.54	
33	PP	13.5	8.8	7.5	8.4			15.0	24.2	23.1	38.7	6.28	
40	IPP	12.8	—	6.5	7.3			7.6	—	21.8	28.4	7.18	
39	IPP	11.8	—	6.7	7.7			8.8	—	21.4	30.0	6.53	
36	IPP	12.9	—	7.5	—			8.2	—	15.4	—	5.27	
38	IPP	13.1	—	6.3	9.6			8.8	—	19.2	24.0	5.30	
Average.....		12.8	—	6.8	7.7			9.2	—	20.6	27.2		

N = Normal.

I = Irradiated.

PP = Partially parathyroidectomized.

IPP = Irradiated and partially parathyroidectomized.

ing figures for the survivors. The fate of these rabbits, therefore, depended not on the primary blood phosphorus level attained shortly after injection, but on the elimination or retention of this excess of

phosphorus in the course of the next few hours. Every rabbit (but one ?) that died had a blood phosphorus level above 21 mg. per 100 cc. serum, 4 to 7 hours after injection. Every rabbit that survived certainly or presumably had a blood phosphorus level below 21 mg. at the same period.

With reference to the occurrence of acute tetany, it is essential that the corresponding figures for blood calcium be taken into consideration. The 2 hour specimens showed a marked drop in blood calcium from the control figures, and this drop was even greater after 4 hours and often reached levels commonly associated with the signs of acute tetany, which all these animals displayed. But it is manifestly significant that differences in the blood calcium level do not distinguish the surviving rabbits from those that succumbed. In the rabbits that died, although the initial drop in blood calcium coincided with the great initial rise in blood phosphorus, the secondary rise in phosphorus that preceded death was in no observed instance accompanied by a further drop in calcium (11), so that just before death occurred the blood calcium in these rabbits was usually somewhat higher than it had been an hour or more previously. Low calcium alone cannot be held responsible for the death of these rabbits.

In the first paper of this series we developed the hypothesis that absolute figures for serum calcium or serum phosphorus are not so significant in experimental tetany as is the ratio between the two elements in the blood. Howland and Kramer (8, 12) have pointed out that in normal children this ratio is approximately 2:1, a proportion most favorable to bone formation, and that in rickets changes in the ratio occur, which may explain, in part, the failure of calcium phosphate deposition. Normally in rabbits also the ratio Ca/P is greater than 1. Among 103 rabbits the average ratio was 1.9:1. In these phosphate-injected animals, however, especially in the instances of phosphate retention in the blood, the ratio fell to a small fraction of its former value. These low ratios are obviously of more significance than are the individual calcium levels alone in interpreting the occurrence of severe and lethal tetany.

In considering the significance of these relative changes in Ca and P concentration, the fact that considerable amounts of the mono-

valent sodium ion were also injected probably must be taken into account. One well established theory of hyperirritability and tetany is based on disturbances in balance between monovalent and divalent cations and anions in the blood. In a very sane and illuminating review and discussion of this question Hastings and Murray (11) weigh the relative importance of monovalent cations and divalent anions as stimulants to nerves. When disodium hydrogen phosphate is injected, a "double dose," so to speak, of nerve irritants is given, and it is hardly profitable to speculate as to which is the chief offender.

It is not our intention on the basis of these few experiments and those reported in the first paper of this series to undertake a critical discussion of current theories of the cause, or more probably causes, of experimental tetany. This vexed and complicated question is not germane to the original object of this investigation. That specific object was attained in the discovery that no significant differences were observable in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or of disodium hydrogen phosphate.

SUMMARY.

No significant differences were found in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or disodium hydrogen phosphate:

Intravenous injections of calcium chloride caused only a transient rise in blood calcium, which returned to its former level within a few hours. The upper level of blood calcium concentration is independent of parathyroid control.

Subcutaneous injections of disodium hydrogen phosphate caused, in all three groups of rabbits, a similar depression of the blood calcium level. After large doses of the phosphate solution, all of the rabbits showed signs of acute tetany in which many of them died. Death or survival was not determined, apparently, by the absolute depression of blood calcium. The rabbits that died were those, in all three groups, in which a phosphorus retention carried the initial rise in serum phosphorus to even higher levels in the hours preceding death, and so changed the normal calcium-phosphorus ratio to a small fraction of its former value.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROIDECTOMIZED RABBITS.

III. THE EFFECTS OF INANITION.

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In order to test the functional significance of the parathyroid hypertrophy due to ultra-violet irradiation of normal rabbits (1) we have subjected irradiated rabbits to partial parathyroidectomy (2) and have compared the effects of injections of calcium chloride and of disodium hydrogen phosphate in irradiated, normal, and partially parathyroidectomized animals (3).

A third method of questioning the significance of this parathyroid hypertrophy was to observe the effects of complete inanition in irradiated, normal, and partially parathyroidectomized¹ rabbits to determine possible differences in the fasting metabolism of calcium and inorganic phosphorus with which the parathyroid glands, directly or indirectly, are so essentially concerned.

Much of the recent work on the physiological and therapeutic effects of ultra-violet light has been devoted to studies on various aspects of metabolic activity, especially with diets which are vitamine-poor, or incomplete in other respects. A fasting experiment with irradiated rabbits also promised opportunities for observation of certain effects of ultra-violet light on metabolism and further information in regard to the action of radiant energy on the endocrine glands.

Accordingly two experiments were undertaken, in which different groups of irradiated, normal, and partially parathyroidectomized rabbits were subjected to fasts of 5 and 11 days, respectively. The

¹ All operations for removal of parathyroids were performed under full ether anesthesia.

5 day period proved too short to yield results of definite significance, and was complicated by the ingestion of unknown amounts of sawdust and feces from the individual cages, so it will not be reported in detail. In outline the experiment ran as follows:

Experiment 1.—This experiment was started with fifteen adult male rabbits in three groups. Five served as normal controls. Five had been irradiated for 44 days with a quartz mercury arc at 1 meter's distance with daily exposures of 10 minutes to 2 hours. The 2 hour exposure was continued during the fasting period. The other five rabbits had been partially parathyroidectomized with the removal of both external parathyroids and half the thyroid gland with its internal parathyroid 50 to 34 days previously. Except in one instance the remaining internal parathyroid gland in these rabbits was maintaining a calcium level of 12.0 to 13.4 mg. per 100 cc. serum. The exception, a rabbit with a control blood calcium of 9.3 mg. and inorganic phosphorus of 8.4 mg. per 100 cc. serum, died on the 5th day of the fast with a blood Ca of 5.7 mg. and P of 12.0 mg. per 100 cc. serum. The rabbits were kept in their regular sawdust-strewn individual cages and given 100 cc. of distilled water by stomach tube daily. Blood samples were taken from the ear before the fast, on the 2nd and 5th days, and 2 to 4 days after feeding had been resumed. The serum specimens were analyzed for Ca (4) and inorganic P (5).

One of the irradiated rabbits was killed by accident on the 5th day of the fast. Autopsy revealed a certain amount of hair, fecal matter, and sawdust in his stomach. Presumably the other rabbits also had ingested indefinite amounts of sawdust and feces, so that the results of this experiment are not open to a strict interpretation. But the average figures for each group show certain tendencies in calcium and phosphorus metabolism that lend emphasis to the results of the second experiment.

Eliminating from the averages for each group the figures obtained from the two rabbits that died and from two others, one normal and one partially parathyroidectomized, for which our records are incomplete, the results of this experiment are shown graphically in Chart 1.

A primary effect of fasting may be seen in the rapid fall in blood calcium that occurred in all of the rabbits. As might be expected, the total loss of calcium by the partially parathyroidectomized rabbits was somewhat greater than that in the other groups. In the first few days after feeding was resumed the blood calcium was restored almost as rapidly as it had been lost during inanition. The more rapid rate of calcium accumulation in the irradiated and partially parathyroidec-

tomized rabbits, as compared with the normals, may be due to a more active state of their parathyroid tissue.

A reciprocal relationship between calcium and inorganic phosphorus may be illustrated by the inverted curves for phosphorus in the irradiated and normal rabbits. Inorganic phosphorus in the blood serum was above normal in these animals during the fasting period and dropped again when the calcium supply was restored. The partially parathyroidectomized rabbits, on the other hand, lost both calcium and phosphorus from the blood;—except in the instance noted above (and not included in the average figures), in which a drop in calcium

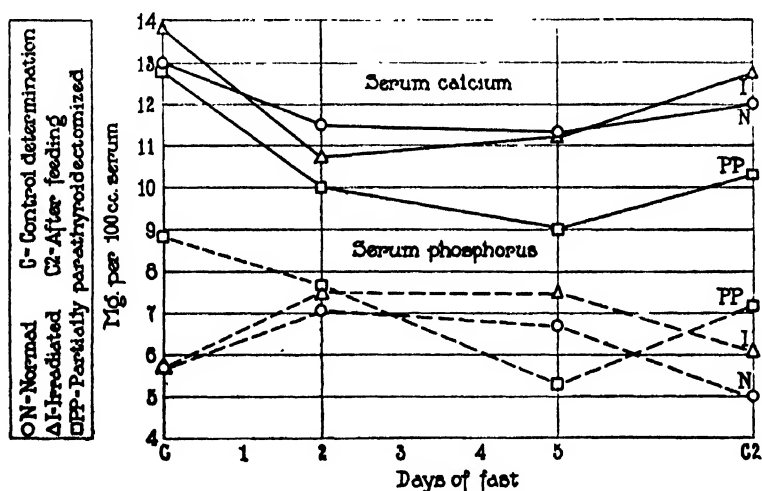


CHART 1. Experiment 1. Serum Ca and P during inanition in normal, irradiated, and partially parathyroidectomized rabbits.

to 5.7 mg. and a rise in phosphorus to 12.0 mg. per 100 cc. serum preceded the rabbit's death.

During this short fasting period none of the rabbits suffered an undue loss in body weight and the average losses for all three groups was less than 10 per cent.

After the final blood samples were obtained, the normal and the irradiated rabbits were killed and autopsied to determine the relative size of their external parathyroid glands. The two external glands of the irradiated rabbits averaged 8.1 mg. per kilo net body weight; those of the normal rabbits averaged 5.7 mg.

In the second experiment the individual metal cages were fitted with raised bottoms of wire mesh through which the urine and feces could fall freely, so that the rabbits ingested nothing but distilled water during the fast.

Experiment 2.—This experiment was performed with fifteen adult male rabbits in three groups. Five rabbits served as normal controls. Five rabbits with ears shaved and backs clipped had been irradiated 2 to 22 minutes daily (except Sundays) for a period of 20 days at 50 cm. distance from a 67 volt, 5.5 ampere quartz mercury arc. During the fast, daily exposures of 23 to 30 minutes were given. The other five rabbits had been deprived of their two external parathyroid glands 10 days previously. The three groups of rabbits were subjected to an 11 day fasting period.

The rabbits were each given 100 cc. of distilled water daily by stomach tube, and were bled from an ear vein for serum samples before the fast, on the 4th, 7th, and 11th days of inanition, and, after feeding had been resumed, on the 12th, 15th, and 18th days of the experiment. The serum samples were analyzed for calcium and inorganic phosphorus. The rabbits were weighed on alternate days.

Two rabbits, one irradiated and one partially parathyroidectomized, died on the 9th day of the fast, and since the figures for Ca and P obtained from them have been excluded from the averages in Chart 2 they must be dealt with separately. The irradiated rabbit started the fast with a serum Ca of 12.7 mg. and P of 8.8 mg. per 100 cc. serum. On the 7th day its serum Ca and P were 8.7 mg. and 10.7 mg. respectively. In 9 days of inanition this rabbit lost 52 per cent of its original body weight and its death is ascribed to causes associated with this great loss, rather than to tetany. The partially parathyroidectomized rabbit had not restored its blood calcium as promptly as is usual after such an operation (2) and started the fast with a serum calcium of 8.9 mg. and phosphorus of 8.8 mg. per 100 cc. serum. On the 4th day serum Ca was 7.2 mg. and on the 7th day 4.9 mg. The corresponding figures for P were 11.3 mg. and 12.7 mg. respectively. Although the death of this animal was not observed, such figures are typical of acute tetany.

Three of the four irradiated rabbits that survived the actual period of inanition died within the next 5 days. The normal and partially parathyroidectomized rabbits were allowed to survive in order to study the period of their recovery. 2 months later a second group of five normal rabbits was subjected to a similar fast and killed at its conclusion to provide a comparison at autopsy with the endocrine glands of the irradiated rabbits. In Chart 2 the figures for Ca and P in the serum of this second group of normal fasting rabbits have been averaged in the curves marked N2.

The figures for calcium and inorganic phosphorus (in mg. per 100 cc.

serum) in the blood of the rabbits which survived the actual period of inanition have been averaged for each group in Chart 2. With few exceptions these averages fairly represent the individual calcium findings. A bizarre and unexplained figure of 15.1 mg. for serum Ca in one of the irradiated rabbits, and a single high Ca of 12.45 mg. in one of the partially parathyroidectomized rabbits on the 4th day of the fast disturb the downward trend of the calcium averages, and have

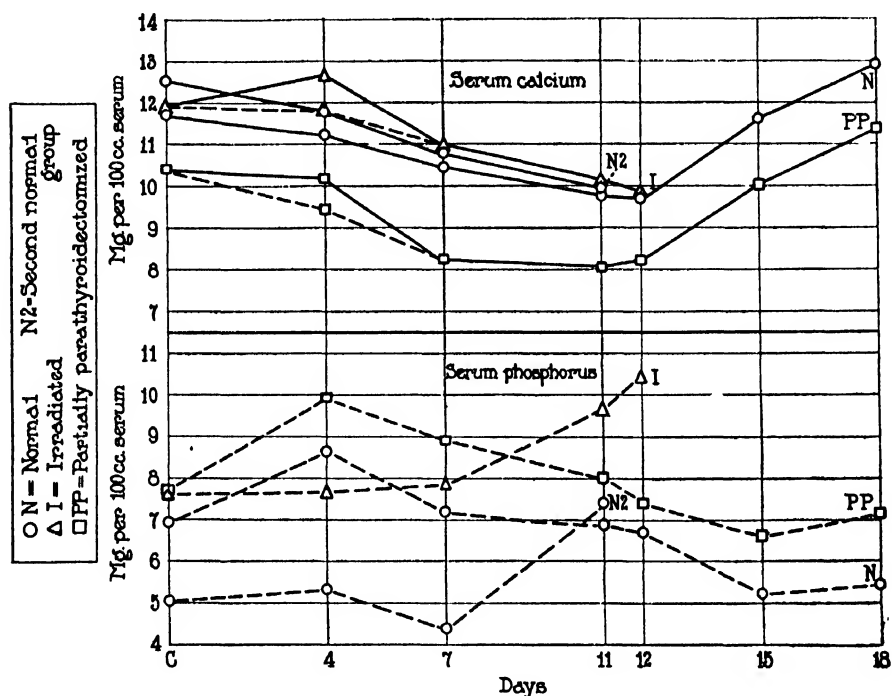


CHART 2. Experiment 2. Serum Ca and P during inanition and after feeding in normal, irradiated, and partially parathyroidectomized rabbits.

been omitted in the alternate averages for that day, also shown in the chart. The corresponding figures for phosphorus in these two animals were not different from those of other rabbits in the same groups.

As in the first experiment, inanition caused a progressive loss in serum calcium in all three groups of rabbits. A similar loss in serum phosphorus did not occur. We shall not attempt to interpret this tendency to phosphorus retention. Wide variations occurred in the figures for the individual rabbits and the averages merely cancel these

variations, so that no group stands out as manifestly different from the others in regard to phosphorus metabolism during the fast. Yet the fact that inanition does not regularly cause a progressive drop in the inorganic phosphorus of the blood serum is shown by the maintenance of the phosphorus levels in six of the seven groups in Experiments 1 and 2.

In regard to serum calcium and inorganic phosphorus metabolism, then, only one difference appears which can be interpreted as due directly to differences in parathyroid function among the four groups of rabbits. The lower level of serum calcium in the partially parathyroidectomized rabbits was maintained throughout both experiments. But the trend of the calcium level in this group was not different from that in the others.

Mention has been made of the fact that one irradiated rabbit died on the 9th day of the fast and that three of the four survivors died within 5 days after feeding was resumed. With the single exception of the partially parathyroidectomized rabbit that died on the 9th day, apparently of tetany, no similar fatalities occurred among the other groups. In general the 11 day period of inanition was well borne by all but the irradiated rabbits. The cause of the deaths in this group must be sought among the direct or indirect effects of the exposure to ultra-violet light. The dosage used, 5 to 30 minutes at 50 cm. distance, is not excessive and had been well borne by normal rabbits. Although the serum phosphorus was high in three of the four victims, serum calcium was not unduly low in the last determination made before death.

Aside from its hypertrophic effect on the parathyroid glands ultra-violet light has two possible effects on the animal which must be taken into account in this experiment. One is on general metabolism. The other is adrenal injury.

A comparison of the relative loss of weight by the three groups of irradiated, normal, and partially parathyroidectomized rabbits shows that from the first the irradiated animals lost weight more rapidly during inanition than did the other groups and toward the end of the fasting period this loss was exaggerated. Even during the first 4 or 5 days after feeding was resumed the weight of most of these rabbits remained stationary or declined further, and it was during this period

that three of the four surviving irradiated rabbits died, each at the nadir of his body weight curve. While the five normal rabbits lost an average of 31 per cent in body weight, and the four surviving partially parathyroidectomized rabbits lost 29 per cent, the five irradiated rabbits lost 52, 41, 39, 48, and 38 per cent of their control weights, respectively, and the first four of these rabbits died on the 9th, 12th, 13th, and 16th day of the experiment.

If loss in body weight be taken as an index of the metabolic rate in these rabbits, the effect of exposure to the quartz mercury lamp is at once apparent. In judging the effects of the exposure two factors have to be taken into account. One is the direct action of visible and ultra-violet light. The other is the handling and the movements of the rabbits due to the daily exposure. All of the rabbits were brought to the laboratory daily for water by stomach tube. In addition, the irradiated group was placed for about half an hour in a galvanized iron tub in which movement was not so restricted. In general they remained quiet, and we do not believe that this extra handling of the irradiated rabbits can account for their excessive losses of weight and for their deaths.

A review of the literature on the effects of visible and ultra-violet light on basal metabolism indicates that experimental conditions and methods have varied so widely that few useful comparisons can be made.

Thus Kestner, Peemöller, and Plaut (6), using a quartz mercury arc lamp in a study with the Benedict apparatus, produced a transient rise in metabolism in children and adults, but Hill and Campbell (7) call attention to the effects of cool air during exposure and consider that the rise in the metabolic rate caused by heliotherapy *per se* is relatively insignificant. Fries (8) found no appreciable difference in basal metabolism of children during treatment with a quartz mercury lamp compared with intervals without treatment, but thinks that the reaction of the individual must be taken into consideration. Recently Campbell (9) has reported a carefully planned and executed series of experiments with rats and mice in a specially adapted metabolism chamber. The oxygen consumption and the carbon dioxide output of four men were tested also, and the author concludes that irradiation with the whole or part of the spectrum of a quartz mercury vapor lamp has no effect on the metabolism of healthy men, rats, or mice. This is a sweeping statement in view of the fact that only the immediate or current effects of the radiation were investigated.

Northrop (10) in a precise experiment with aseptic cultures of *Drosophila*, grown for 200 generations in the dark, found that the light of a tungsten filament

lamp, through water, had marked effects on growth and duration of life. With intensities greater than 1000 meter candles the duration of the imago stage was rapidly shortened and the duration of life of the imago could be predicted by assuming that the light ages the flies independently of the normal rate. The light effect was proportional to its intensity.

In the absence of observations on basal metabolism in our groups of fasting rabbits, we can only call attention to the excessive loss of weight in the irradiated rabbits, and suggest its possible relation to an increased metabolic rate resulting in the more rapid consumption of the animal's own tissues. A definite effect of fasting and the exposures to radiant energy was found at autopsy in the irradiated victims of the fast.

From time to time we have observed gross hemorrhages in the suprarenal glands of irradiated rabbits (1). The point of origin of these hemorrhages has usually been the medullary tissues, but they have often extended into the cortex and even to the capsule of the affected gland. The frequency of such a lesion after irradiation, even when only moderate erythema doses have been given, has raised in our minds the question of a specific secondary action of ultra-violet energy on these essential organs. The effects of such an injury might be reflected clinically in blood pressure changes, which were not studied in these fasting rabbits. Under other conditions, light, especially ultra-violet light, has often been observed to lower blood pressure (11) and the signs of collapse that sometimes follow dangerous degrees of sunburn are in certain respects similar to the signs of suprarenal insufficiency (12).

According to Jackson (13), and other authors whom he quotes, inanition and various types of malnutrition often cause suprarenal congestion and hemorrhage. In fasting the suprarenals do not show a proportionate loss in weight, sometimes being relatively twice as large as is normal. Congestion, and sometimes hemorrhage, is characteristic in both cortex and medulla and may account for the increased weight in spite of atrophy of the parenchyma. Congestion and hemorrhage, gross or capillary, have been reported also in athrepsia, scurvy, and pellagra, so it appears that the suprarenal glands often suffer heavily in states of malnutrition.

With two causes of suprarenal damage operating in conjunction, it

is hardly surprising that three of the four irradiated rabbits examined at autopsy showed scars and medullary hemorrhage in one or both of these essential glands. One gland of the fourth rabbit was lost, and its condition cannot be reported. The other gland appeared to be normal. None of the lesions were so recent as to suggest that they were agonal, or the immediate cause of death, but suprarenal insufficiency may have contributed to the high mortality among the irradiated rabbits.

TABLE I.

Glands	Gland weights (in mg. per kg. net body weight)				Change in gland/body weight ratio, effect of					
	Normal		Irradiated		Irradiation		Fasting		Both	Fasting
					I	II	III	IV	V	VI
	Controls	Fasted	Controls	Fasted	IC/NC	IF/NF	NF/NC	IF/IC	IF/NC	Jackson
Pineal.....	6.7	11.3	10.2	14.1	+	+	+	+	+	±
Hypophysis.....	14.8	18.2	16.9	29.6	+	+	+	+	+	+
External parathyroids.....	6.1	5.5	9.2	7.0	+	+	-	-	+	-
Thyroid.....	112.8	116.4	92.2	156.2	-	+	±	+	+	+
Thymus.....	1151.	390.	1111.	402.	±	±	-	-	-	-
Suprarenals.....	195.0	279.7	212.5	374.6	+	+	+	+	+	+
Testicles.....	2828.	3122.	2670.	3066.	±	±	+	+	+	-

N = Normal. I = Irradiated. C = Control. F = Fasted.

+ = Gland spared. Weight of gland increased relative to body weight.

- = Gland consumed. Weight of gland decreased relative to body weight.

At autopsy other endocrine glands of the fasted, irradiated rabbits, and of the fasted, normal rabbits of Group N2, were weighed and their weights compared with figures obtained for other irradiated and normal rabbits at a corresponding season of the year. These figures and a crude analysis of them have been collected in Table I, which may require some explanation.

The figures given represent gland weights, in mg. per kilo of net body weight, after the removal of the gastrointestinal tract and its contents, and the expression of bladder urine. For the normal control figures we are indebted to Dr. Louise Pearce. In the second half

of the table an attempt has been made to indicate whether fasting spares these essential glands, or exhausts and consumes them more rapidly than other body tissues. The effect of irradiation alone is seen in the gland weight ratios of irradiated and non-irradiated rabbits in both the control and the fasting groups. An idea of the effect of fasting alone is gained by comparing fasted with control rabbits in the normal and irradiated groups. A summation of the effects of irradiation and fasting is found in a comparison of the figures for the irradiated fasted rabbits with the normal controls. Considering the small number of fasted rabbits available for study, the results of the analysis are in good accord. The effects of irradiation alone, Columns I and II, check in most instances both for normal and for fasted rabbits and in the control, irradiated, rabbits the same effects of irradiation are seen as have already been reported (1): pineal, hypophysis, external parathyroid, and suprarenal glands are relatively heavier than normal, the thyroid gland has lost weight, and the thymus and testicles show little change. Similarly the effects of inanition on these endocrine glands may be seen in Columns III and IV. Pineal, hypophysis, thyroid, suprarenals, and testicles are spared in both groups of rabbits. The external parathyroids, and especially the thymus are consumed relatively faster than the body as a whole. Finally, in the single instance in which the effects of fasting and of irradiation on gland weight seem to be opposite, *i.e.* the effect on the external parathyroid glands, the light treatment has more than offset the effects of fasting, and the parathyroids in the irradiated fasted rabbits are larger than is normal. But in spite of the relatively large size of the external parathyroid glands in the irradiated rabbits, their curves for serum calcium during the fasting period in both experiments are essentially similar to those for the normal groups (see Charts 1 and 2).

In most instances these tendencies to the protection or exhaustion of the endocrine tissues in our fasted rabbits correspond with the effects of inanition in man and other animals, reviewed and summarized by Jackson (13). His general conclusions in regard to each gland are indicated in Column VI in the table, and may be noted briefly as follows:

Pineal Gland.—No appreciable change relative to body weight. No conclusions drawn.

Hypophysis.—Loss of weight relatively less than that of the body as a whole.

Parathyroid Glands.—Usually found to be atrophic. In rats Jackson found them reduced in weight nearly in proportion to the body loss. They tend to be relatively decreased in weight in adults, with a variable degree of atrophy and an increase in fibrous stroma.

Thyroid Gland.—The loss of weight is variable, usually relatively less than that of the body as a whole.

Thymus.—The extreme loss of weight of this gland is rivaled only by that of adipose tissue. The loss is relatively far greater than that of the body and usually reaches 75 per cent before death from acute inanition.

Suprarenals.—These glands are usually increased in weight but part of this increase may be due to hemorrhage.

Testicles.—The loss in weight is relatively greater than that of the body as a whole.

Thus the only result of our observations that is at variance with Jackson's conclusions is in the loss of weight of the testicles relative to general body weight loss. But these glands normally vary widely in absolute and relative weight, and we lay no stress upon the aberrant results of our single experiment.

SUMMARY AND CONCLUSIONS.

The experiments described in the three papers of this series were undertaken to determine if possible the significance of the parathyroid hypertrophy that has repeatedly been found in rabbits after exposure to ultra-violet light. The fact that the hypertrophy was not accompanied by a corresponding increase in blood calcium led us to infer that the upper level of blood calcium concentration is governed by factors independent of parathyroid control, and that the gland hypertrophy under the influence of ultra-violet light might represent a potential functional capacity, or increased factor of safety, to protect the calcium level under conditions of stress or emergency. Partial parathyroidectomy in irradiated rabbits, compared with the same operation in normal controls, resulted in a similar immediate drop in blood calcium; but a more rapid restoration of the calcium level in the irradiated rabbits indicated a more prompt and active response to the emergency on the part of the remaining glands. In the second group

of experiments no significant differences were found in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or of disodium hydrogen phosphate. All three groups of rabbits rapidly eliminated the excess calcium from the blood. Their reactions to large doses of sodium phosphate, given subcutaneously, appeared to be related to phosphate elimination or retention in the individual animals. Those in each group which had phosphate retention in the blood died in acute tetany.

So also in the fasting experiments, reported in this paper, no distinctions could be drawn between normal, irradiated, and partially parathyroidectomized rabbits on the basis of their calcium and phosphorus metabolism. Blood calcium was reduced in a similar manner in all three groups of rabbits, and wide individual variations in serum phosphorus levels preclude significant deductions.

But the irradiated rabbits suffered disproportionate losses of weight during the fasting period and four of five of them died during the course of the experiment. The deaths of these rabbits are attributed to this more rapid consumption of their own tissues and possibly also to suprarenal injury caused both by fasting and by exposure to ultra-violet light.

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THE ETIOLOGY OF VERRUGA PERUANA.

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PLATES 2 TO 4

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The experimental investigation to be reported in this paper was made possible through the cooperation of Professor Oswaldo Herccelles, of the University of Lima, who was kind enough to obtain for me selected material for study, and I wish at the outset to acknowledge my indebtedness to him and my appreciation of his courtesy. I wish to express my thanks also to Professor E. Campodónico, of the University of Lima, through whose courtesy the material was forwarded from Lima to New York.

There are many similarities between the conditions known as verruga peruana and Oroya fever. Their geographical distribution, which is rather curiously limited to certain narrow valleys on the western slopes of the Andes Mountains, between 6° and 13° south latitude, is practically the same and is of the type shown by infections conveyed by certain ectoparasites. Fever and anemia are considerably more acute and severe in Oroya fever, but both are present in verruga. Verruga is predominantly a chronic infection, and its conspicuous feature is the characteristic nodular eruption on the skin which lasts for a period of a few weeks to one of many months. Oroya fever, on the other hand, while sometimes accompanied or followed by a similar skin eruption, is distinguished by an acute course of extreme anemia, during which a specific intracellular parasite, *Bartonella bacilliformis*, is abundantly present in the red blood corpuscles. This microorganism has rarely been seen in the blood in cases of benign verruga.

In 1910 Jadassohn and Seiffert¹ showed that verruga peruana could be transmitted to monkeys by local inoculation of suspensions

¹ Jadassohn and Seiffert, G., *Z. Hyg. u. Infektionskrankh.*, 1910, lxvi, 247.

of tissue from human verruga lesions, and their results have received confirmation from Mayer, Rocha-Lima, and Werner,² and from the Commission of the Harvard School of Tropical Medicine.³

Oroya fever has not thus far been directly transmitted to animals, but by means of a culture recently isolated from a fatal case of the disease⁴ it has been shown that young *rhesus* monkeys (about 2,000 gm. body weight) are susceptible to infection with *Bartonella bacilliformis*, though usually not to the same degree as man, and that in the infected animals the parasite is demonstrable in its characteristic situation in the erythrocytes. In some animals which are less resistant to infection, the extreme anemia of Oroya fever has been reproduced.⁵ The striking fact brought out in the inoculation experiments, however, was the dual nature of the infection induced, for characteristic signs of both Oroya fever and verruga were observed in the animals, with occasionally a marked favoring of one or the other type of disease, as the virulence of the strain became enhanced by adaptation to the experimental animal. Systemic manifestations—moderate or marked anemia, fever, and high titer of *Bartonella bacilliformis* in the blood, bone marrow, lymph nodes, and spleen—were in general more severe in animals inoculated intravenously or intraperitoneally, and death occurred only in animals so inoculated, but the invasion of the blood by the parasite was found to take place also after local inoculation. The tendency toward the production of verrucous lesions was more marked, as a rule, when the virus was introduced locally, and the lesions usually arose only at the sites of inoculation, as is the case when monkeys are inoculated directly with human verruga material.^{1,2} In one instance, however, in which passage virus was injected both locally and intravenously, a spontaneous general eruption typical of severe human verruga arose on various parts of the skin, remote from the sites of inoculation. The skin lesions of the animals, whether local or

² Mayer, M., Rocha-Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

³ Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaturú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

⁴ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

⁵ Noguchi, H., *J. Exp. Med.*, 1926, xlv, 697.

spontaneous in occurrence, in all instances yielded cultures of *Bartonella bacilliformis*, and stained sections of all such lesions revealed the parasites in large numbers in a characteristic endothelial situation.⁶

These findings, when taken in relation to the historical and epidemiological data with regard to the two conditions,⁶ suggested very strongly that Oroya fever and verruga peruana were both due to *Bartonella bacilliformis*, the considerable variations in the manifestations of infection being a result of the differences in resistance among individuals or among different tissues of the same individual. It was desirable, however, to approach the problem of the etiology of verruga directly, by means of the cultural and experimental methods used for the isolation of *Bartonella bacilliformis* from Oroya fever.

For the purpose of comparative study subcutaneous nodules were removed from each of two cases of verruga in the Dos de Mayo Hospital, Lima, placed in tubes, and covered with citrate-saline solution (2 per cent sodium citrate in 0.9 per cent sodium chloride). The tubes were sealed, abundant air space being allowed, and were placed in the ship's refrigerator during the journey to New York, a period of 14 days. We were fortunate in having transportation so arranged that the tissues were shipped shortly after their excision from the patients and were received in the laboratory the same day that they arrived in New York (April 7, 1926). Each specimen was immediately examined microscopically, saline suspensions were made, and inoculated into culture media and experimental animals, and a portion of each tissue was fixed in Regaud's fluid for histological study.

The nodule of Case P. 5 measured about $8 \times 12 \times 6$ mm. and was light pinkish in color, with the exception of a small portion to which the skin was still attached. The nodule was still firm, except for slight softening along the cut surfaces. The citrate solution was slightly turbid, owing to liberation of tissue elements, but there was no putrefactive odor.

Minute Gram-negative bacilli were present, occurring singly, in pairs, and in larger groups, as well as in masses (Fig. 18). Sections stained with Giemsa's and Gram's solutions showed masses of the organisms scattered through the tissue (Fig. 18), the general structure of which was still well preserved (Fig. 17) notwithstanding loss of cellular elements through autolysis.

⁶ Odriozola, E., La maladie de Carrion, Paris, 1896.

The nodule from Case J. 45 was not as well preserved. The tissue was soft and friable, but there was no sign of bacterial putrefaction. It contained numerous Gram-negative bacilli similar to those found in the specimen from Case P. 5 and in addition a few small Gram-positive diplococci. In sections the bacilli were found in the nodular tissue and the cocci near and on the skin.

Two species of minute Gram-negative microorganisms were isolated from the tissue of Case P. 5, one indirectly, from monkeys inoculated with suspensions of the tissue, and one by direct cultivation. The former is pathogenic for young *Macacus rhesus* monkeys and in every respect has proved identical with the strain of *Bartonella bacilliformis* previously obtained from the blood of a case of Oroya fever. The latter, although present also in the J. 45 tissue, as shown by cultivation experiments, is apparently a non-specific invader; it has failed to induce characteristic local or systemic infection, and it grows within 24 hours on ordinary agar slants, while the pathogenic organism grows slowly and only on leptospira medium or blood agar slants. Our interest, therefore, is in the pathogenic strain obtained by inoculation of the P. 5 material.

Transmission of Verruga Peruana to Monkeys with the Excised Nodular Tissue of Case P. 5.

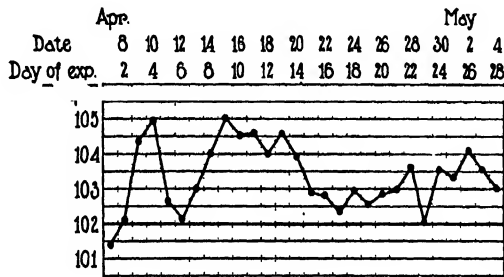
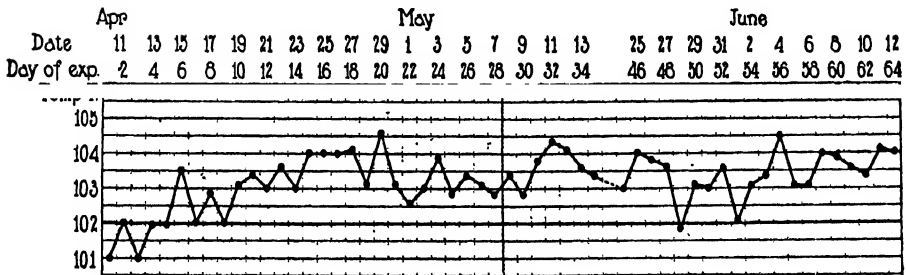
Transmission of verruga peruana to monkeys, as stated earlier in this paper, has repeatedly been accomplished by previous investigators. In the present instance, however, the tissue had been excised from the patients more than 2 weeks previous to inoculation.

One young and one adult *Macacus rhesus* were available on April 7. The young one was used for inoculation of the more promising material, that is, the P. 5 nodule. Another young monkey, obtained on April 10, was also inoculated with the P. 5 material. Owing to the scarcity of suitable animals, the inoculations with the J. 45 material could not be repeated.

The suspension of the P. 5 nodule induced definite systemic or local infection in both animals inoculated; the full grown monkey receiving inoculation of the J. 45 material showed no symptoms of infection.

M. rhesus 33, inoculated Apr. 7, 1926, with a saline suspension of the nodule P. 5, intradermally on the shaved right eyebrow and by scarification on the left eyebrow.

2 cc. of the suspension were also injected into the saphenous vein of the right leg. After 48 hours there was a rise of temperature (Chart 1) which lasted for 2 days, but blood taken at this time yielded no growth. On Apr. 14 the temperature rose again and remained high for 6 days. Blood taken during the fever (Apr 16) and inoculated into leptospira medium yielded, in a 1:10 dilution, pure growth of a microorganism indistinguishable from *Bartonella bacilliformis*. The sites of intradermal inoculations on the eyebrow showed some induration at this time, but they never increased in size and receded within a few weeks. The lymph glands in the inguinal and axillary regions became markedly enlarged about Apr. 16. Blood cultures made on two successive occasions, May 27 and June 20, 1926, both gave pure cultures of the *Bartonella*-like organisms.

CHART 1. *M. rhesus* 33.CHART 2. *M. rhesus* 34.

M. rhesus 34, inoculated Apr. 10, 1926, with the same material and in the same way as *M. rhesus* 33. A moderate rise of temperature occurred 14 days from the time of inoculation (Chart 2), and the animal remained febrile for 4 days. Blood cultures made on Apr. 28, 1926, yielded a pure growth of the same organism as had been isolated from *M. rhesus* 33. At this time the lymph glands had become markedly enlarged, but no local reactions were noticeable. Blood taken on May 12 and on May 27 was culturally positive in a 1:10 dilution. At the beginning of June a large subcutaneous nodule at the middle portion of the tail was noticed

(Fig. 10), and on June 18 it was excised.* It showed the typical histology of verruga tissue (Fig. 11) and yielded a pure culture of the same organism as had been obtained from the blood. Cultivation of the blood was again successful on June 21, this time with a dilution of 1:100.

The foregoing experiments demonstrate the infectivity of the verruga nodule which had been kept at refrigerator temperature for at least 2 weeks, and the association of infective power (as evidenced by febrile reaction and the production of a metastatic local verruga lesion) with a definite microorganism recoverable by culture both from the blood of the animals and from the local lesion. Blood counts revealed no anemia in either animal, and in neither instance did the blood films contain a sufficient number of organisms to be detected microscopically.

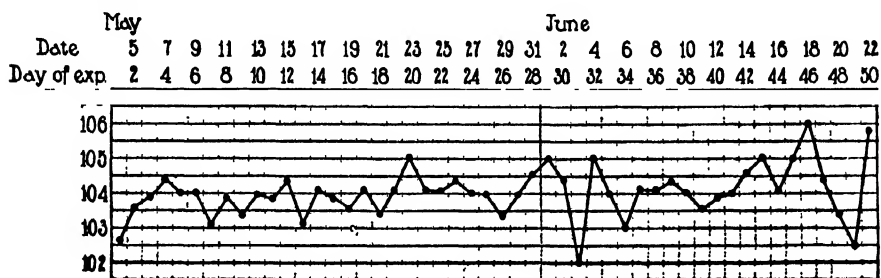


CHART 3. *M. rhesus* 41.

The pathogenicity of the culture obtained from the blood of *M. rhesus* 33 on April 16 was tested by inoculation of *M. rhesus* 41. As the protocol shows, the culture gave rise not only to a local reaction but to a systemic infection of pronounced severity.

M. rhesus 41 (Fig. 1) inoculated May 4, 1926, with culture derived from the blood of *M. rhesus* 33 and grown for 18 days on leptospira medium. The material was inoculated on the right eyebrow and abdomen intradermally and on the left eyebrow and abdomen by scarification. An irregular remittent febrile reaction (Chart 3) began to be manifest 72 hours from the time of inoculation and continued during the 2 months of observation. Induration became noticeable at the site of intradermal inoculation on the right eyebrow after 14 days. The lesion had the gross appearance of a typical verruga nodule, and histological examination of the

* All operations were carried out under ether anesthesia.

excised tissue showed the characteristic granulomatous structure (Figs. 4, 12) and the presence of bacilliform organisms in the endothelial cells (Fig. 13). A suspension of the nodular tissue yielded pure cultures of the organisms in a 1:10 dilution, as did also blood withdrawn on May 21. On May 27 the blood titer by culture was 1:10,000, and on June 3, 1:100,000. The nodules on the eyebrow rapidly increased in size, becoming scarlet-red and protruding (Fig. 1). Blood films made on June 3 showed numerous bacilliform bodies in the red corpuscles, the organisms being present in the largest numbers so far observed in *rhesus* monkeys (Figs. 2, 14). Small nodules developed at the sites of intradermal inoculation on the abdomen 40 days after inoculation; at that time the nodules on the eyebrow measured about 10×12 mm.

Blood counts made by Dr. J. H. Bauer showed a gradual diminution of red corpuscles and hemoglobin and an unusually high leucocytosis:

	Erythrocytes per c.mm.	Hemoglobin	Leucocytes per c.mm.
		per cent	
May 4 (day of inoculation).....	5,088,000	80	
" 14 (10 days after inoculation).....	4,632,000	70	
June 3 (30 " " ").....	4,760,000	55	17,600
" 15 (42 " " ").....	4,800,000	40	23,000
" 23 (50 " " ").....	3,392,000	30	43,000
" 25 (52 " " ").....	3,460,000	25	40,000
" 28 (55 " " ").....	3,552,000	25	37,800

The reduction in hemoglobin, it will be noted, was relatively greater than that in the number of red cells, a phenomenon so far not observed by us in animals infected with the strain of *Bartonella bacilliformis* from Oroya fever.

Blood smears stained on June 25 revealed a peculiar appearance of the intracorpuseular parasites, which were still present in rather large numbers, but stained reddish rather than violet, appeared thinner, and in some instances were fragmented. The general features of the organisms suggested that they were undergoing degeneration (Figs. 3, 15). Blood taken at this time yielded cultures in dilution of 1:10, but not in 1:100. A week later no intracorpuseular parasites could be demonstrated, the animal was more active, and the nodules had decreased somewhat in size. Conditions seemed to indicate the development of a state of immunity. No cultures could be obtained from the blood after June 25. On Aug. 11 there was still anemia (red blood cells 3,362,000, hemoglobin 50 per cent), but on Sept. 14 the number of red cells had increased to 4,336,000.

The nodule excised on May 19 and the blood withdrawn 2 days later were inoculated into two *rhesus* monkeys. As the protocols show, both materials gave rise to local lesions and also to systemic infection.

M. rhesus 42, inoculated May 19, 1926, with a saline suspension of the nodule from the eyebrow of *M. rhesus* 41, on the left eyebrow and left abdominal wall by intradermal injection and on the right eyebrow and right abdominal wall by scarification. This animal had fever for only a few days (Chart 4) during the 45 days of observation, but cultures made with the blood on June 2 and 14, 1926, gave pure growth of the bacilliform organisms in dilutions of 1:10 and 1:1,000 respectively.

Small reddish indurated areas along the lines of scarification on both eyebrow and abdomen became noticeable on June 1 (Fig. 6), and 10 days later the scarified areas appeared as linear rows of eruptions (Figs. 7, 8). Within another 10 days the adjacent lesions had become confluent (Fig. 9) but the transverse lines were still well separated. The intradermal inoculations in this instance, contrary to the usual case, failed to induce any lesions.

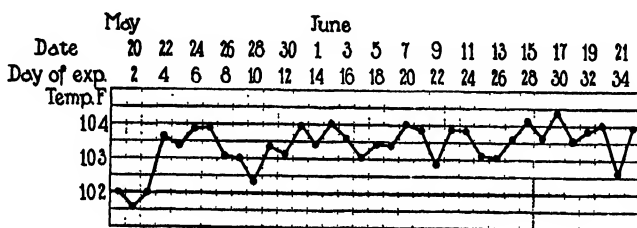


CHART 4. *M. rhesus* 42.

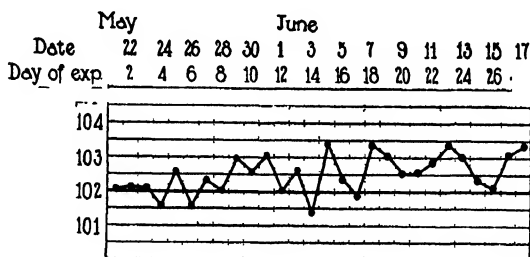


CHART 5. *M. rhesus* 43.

The lesions had all healed by Aug. 6, 1926.

M. rhesus 43, inoculated May 21, 1926, under ether anesthesia, with the citrate blood withdrawn on the same day from *M. rhesus* 33. Intradermal inoculations were made on both eyebrows and on both sides of the abdomen. Blood was also applied to scarified areas on the eyebrows and abdomen, and 0.5 cc. was injected intravenously.

The animal showed no febrile reaction at any time during the 45 days of observation (Chart 5). Blood cultures made on June 26 were negative. Small nodules, 2×2 mm., appeared at each of the two sites of intradermal inoculation within about 24 days. They showed no change during the ensuing 10 days but

subsequently gained in size rather rapidly. They were situated subcutaneously, were perfectly round, and had a semitransparent, bluish, pearl-like color. On July 1 there were noticed about the periphery the minute red streaks which *Bartonella* lesions of this type show just before vascularization. One of the nodules excised at this time (July 1) yielded cultures in a dilution of 1:100,000. By Aug. 6, 1926, the lesions had all healed.

The microorganism obtained in culture from a monkey inoculated with human verruga tissue (Case P. 5) was capable, therefore, of inducing in *rhesus* monkeys a local and systemic infection the manifestations of which were in all respects similar to those obtained by inoculation of the strain of *Bartonella bacilliformis* from Oroya fever, *i.e.*, a prolonged course of irregular, remittent fever, during which the intracorpuseular parasites are present in the blood, and a skin lesion characterized by extensive proliferation of endothelial cells and by the presence of the parasites in the cytoplasm of these cells. The infection in the second passage animal (Monkey 41) was of the severe type; there was absolute anemia and hyperleucocytosis, and the local lesions were very large. The organisms were present in large numbers at one period of the disease, but only degenerated forms were found at a later stage. The blood from which the culture was obtained, however, induced only a mild, slowly developing infection, with no fever.

The local inoculation of nodular tissue from Monkey 41 into Monkey 42 (third passage) induced a severe local reaction and a febrile reaction of brief duration, during which the blood culture titer was as high as 1 1,000. As stated previously, direct microscopic detection of the parasites in the blood was unsuccessful in the case of mild infections. The cultural procedure was by far the most delicate means of diagnosis.

Cultural and Morphological Characters of the Microorganisms from Verruga.

The character of the lesions of *M. rhesus* 41 and the microscopic findings in the blood of this animal appear to leave no doubt that the organism injected was identical in pathogenic properties with the strain of *Bartonella bacilliformis* previously obtained from Oroya fever, a conclusion which is further substantiated by cultural and morphological study.

The first culture was obtained on the so called leptospira medium, which was inoculated with various dilutions of citrated blood of *M. rhesus* 33 and kept at 25°C. Growth became recognizable macroscopically within a week, the uppermost layer of the medium showing some grayish, minute particles; these gradually increased in number during subsequent weeks (Fig. 19). Under the dark-field microscope these particles were seen to consist of numerous tightly packed masses of irregular minute bacillary bodies. Individual organisms were occasionally seen, but the chief characteristic of the organisms is their constant tendency to form firm aggregates difficult to disperse (Fig. 21). Free flagella are seen in the agar mass.

On the surface of blood agar round raised colonies of variable size, the smallest almost microscopic (Fig. 20), the largest having the appearance of fine granules, are formed within about 6 to 8 days and after a few days reach 1 mm. or more in diameter, but seldom 2 mm. A light grayish color is noticeable when the light falls obliquely on the culture. The colonies are firm and are peeled off the agar readily by a platinum loop. It is difficult to break them up in fluid to form a uniform suspension. Under the dark-field microscope masses of agglomerated organisms are seen to predominate (Fig. 22); a few single or paired individuals may be found, and these are usually motile. Locomotion is in one direction only, but rotatory movement is also seen.

The organisms are Gram-negative, pleomorphic, and stain unevenly with Giemsa's solution. When stained a long time they appear reddish and ill defined in contour; they stain much less intensely than most bacteria. Special staining reveals two or more flagella attached to one end of the organism (Fig. 24), the length of the normal flagella varying from 2 to 5 μ . Abnormal detached flagella may reach a length of 20 to 30 μ .

Individuals in young cultures are more uniform in size and have a definite contour. Short rods, 0.3 to 0.4 μ wide and 0.45 to 1.5 μ in length, predominate. In older cultures the individuals are much more irregular in size and form, and numerous granular elements measuring less than 0.2 to 0.5 μ are found in masses; these stain intensely and have very indefinite outlines, the appearance simulating that of degenerative changes. Exceptionally long rods 2.5 to 3 μ may be found. There is no polar staining.

The optimum temperature for growth is 25–28°C.; at 37°C. growth ceases within 4 to 5 days. The optimum reaction of the medium lies between pH 7.8 and pH 8.

For the cultivation of this organism the addition of animal blood or serum and hemoglobin to solid or semisolid media is essential. No satisfactory fluid medium has so far been found. The organism is an obligate aerobe. The cotton plugs of culture tubes may be impregnated with paraffin, but sealing them hermetically with sealing wax prevents growth.

Like the strain of *Bartonella bacilliformis* from Oroya fever, the organism is non-spore-forming and hemophilic. It is provided with

TABLE I.

Anti-Oroya serum	Antigens	
	Oroya	Verruga
	0.5 cc.	0.5 cc.
cc.		
0.1	++++	++++
0.01	++++	++++
0.001	++	—
0.0001	—	—
0	—	—

++++ = complete fixation.

++ = 50 per cent hemolysis.

— = complete hemolysis.

several unipolar flagella of the characteristic bacterial type. A distinct cell wall, such as is seen in bacteria, can be recognized in young, actively motile forms.

The organism does not ferment any carbohydrate so far tested. Red corpuscles contained in the culture medium are not hemolyzed in the course of its growth. No putrefactive process seems to occur in the culture. Slight growth may be obtained on Loeffler's serum medium, but no liquefaction is observed.

Serological Identification of the Verruga Strain.

The morphological, cultural, and pathogenic properties of the verruga strain resemble so much those of the strain of *Bartonella*

bacilliformis obtained from a case of Oroya fever that the identity of the two organisms appeared extremely probable. Preliminary serological tests confirmed this conclusion.

A rabbit was repeatedly inoculated intravenously with saline suspensions of cultures of *Bartonella bacilliformis* during a period of 4 months, 1 to 2 cc. being injected at intervals of 4 to 5 days, and the serum obtained from the animal was tested for complement fixation with its homologous strain and also with the strain from verruga. Saline suspensions of colonies removed from the surface of blood slant cultures after 14 days growth at 25°C., and washed twice with saline solution, served as antigens. The suspensions were rather granular in appearance, owing to the difficulty of breaking up the colonies. To 0.5 cc. of the antigen (this quantity did not bind complement) were added the immune serum in quantities of 0.1, 0.01, 0.001, or 0.0001 cc., and 0.04 cc. guinea pig serum as complement. Two hemolytic units of anti-sheep amboceptor were used. Controls with normal rabbit serum were made in each instance. The total volume in each case was made up to 1.2 cc. with saline solution. The results are shown in Table I.

The results of the complement fixation tests show that there is an undeniably close serological relationship between the two strains, although the immune serum reacted more strongly with the Oroya than with the verruga strain.

SUMMARY AND CONCLUSIONS.

A saline suspension of a subcutaneous nodule excised from a verruga patient, and kept in the refrigerator for 14 days, on inoculation into two young *Macacus rhesus* monkeys (Nos. 33 and 34) induced irregular febrile reactions and enlargement of the lymph glands, and in one instance a subcutaneous nodule arose, independently of direct inoculation, on the tail. A microorganism has been isolated from the blood of both animals, and from the experimental nodule, which in pathogenic properties and in cultural and morphological characteristics is indistinguishable from the strain of *Bartonella bacilliformis* isolated from a case of Oroya fever.

The spontaneous skin lesion of Monkey 34 and the subcutaneous nodules induced by intradermal inoculation of cultures of the microorganism were histologically typical of experimental verruga lesions in monkeys and identical with the skin lesions induced in monkeys by *Bartonella bacilliformis*. The organism, like *Bartonella bacilliformis*, is an intracellular parasite, being found in the cytoplasm of the proliferating

erating endothelial cells of the lesions and in the erythrocytes of the blood.

The same variations in the manifestations of disease which have been noted in experimental infection with *Bartonella bacilliformis* were observed in the experimental verruga infection. In the second passage (Monkey 41) the infection induced by local inoculation of cultures was severe both locally and constitutionally and was accompanied by marked anemia. The organisms were found in the red cells in large numbers. In the third passage the systemic infection was less severe, but the local lesions were more striking.

Detection of the parasites in the blood is far more certain by the cultural method than by microscopic examination, the latter procedure being successful only in rather severe infections. The result of blood culture is therefore the decisive method in the final diagnosis of the disease.

Preliminary serological study shows that the organism isolated in the present instance from the skin lesion of a verruga patient and that previously obtained from the blood of a case of Oroya fever belong to the same serological group.

The data obtained justify the conclusion that verruga peruana is caused by *Bartonella bacilliformis*. They also definitely establish the fact that the inoculation of blood or sanguineous exudate from lesions of verruga peruana is capable of inducing in susceptible individuals a severe febrile systemic infection, such as that to which Carrion succumbed. The designation "Carrion's disease"⁶ is therefore the appropriate one for both forms of the infection.

Bartonella bacilliformis may be regarded as a bacterium, since it has the essential features of that group of microorganisms.

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. *M. rhesus* 41, showing three verruga nodules on the right eyebrow 30 days after intradermal inoculation. The two nodules on the inner side had arisen at the site of removal of the initial nodule 16 days previously. Natural size.

FIG. 2. Verruga organisms in the red corpuscles of *M. rhesus* 41, 30 days after inoculation. $\times 1,500$.

FIG. 3. Verruga organisms in the red corpuscles of the same monkey 52 days

after inoculation. They took a more reddish stain at this time and were less definite in outline, probably because they were in process of degeneration. Anemia was also most marked at this time. Subsequent attempts to find the parasite in the blood were unsuccessful. $\times 1,500$.

FIG. 4. Verruga organisms in the nodule of *M. rhesus* 41, removed 14 days after inoculation. Giemsa's stain after fixation in Regaud's fluid. $\times 1,500$.

FIG. 5. Verruga organisms in the nodule of a patient suffering with verruga peruana. Courtesy of Professor R. P. Strong. Giemsa's stain, after fixation in Zenker's fluid. $\times 1,500$.

FIG. 6. Early lesions of experimental verruga in *M. rhesus* 42, 16 days after inoculation of a scarified area on the right eyebrow with a suspension of the nodule from *M. rhesus* 41. Natural size.

FIG. 7. Same lesions 23 days after inoculation.

FIG. 8. Early lesions on the scarified skin of the abdomen of the same monkey 16 days after inoculation. Natural size.

FIG. 9. Same lesions 23 days after inoculation.

FIG. 10. Subcutaneous nodule which arose spontaneously on the tail of *M. rhesus* 34, 59 days after the animal had been inoculated with a suspension of a nodule of the verruga patient P. 5. Natural size. For histological appearance of the nodule see Fig. 11.

PLATE 3.

FIG. 11. Histological appearance of the subcutaneous nodule on the tail of *M. rhesus* 34, 69 days after inoculation. (The gross appearance of the nodule is shown in Fig. 10.) Giemsa's stain after fixation in Regaud's fluid. $\times 152$.

FIG. 12. Histological appearance of the nodule on the eyebrow of *M. rhesus* 41, removed 14 days after inoculation. Giemsa's stain after Regaud's fixation. $\times 152$.

FIG. 13. Verruga organisms in the nodule of *M. rhesus* 41. Same section as shown in Fig. 12. $\times 1,000$.

FIG. 14. Verruga organisms in the red blood corpuscles of *M. rhesus* 41. Eight different fields have been placed side by side. In some of them are seen red corpuscles containing one or more organisms. Blood films made 30 days after inoculation. Giemsa's stain. $\times 1,000$.

FIG. 15. Blood of the same monkey 52 days after inoculation. The organisms appear somewhat degenerated but quite numerous. Giemsa's stain. $\times 1,000$.

FIG. 16. *Bartonella bacilliformis* in the blood of an Oroya fever patient, for comparison (Patient S. A. 15, case from which *B. bacilliformis* was cultivated). Giemsa's stain. $\times 1,000$.

PLATE 4.

FIG. 17. Verruga nodule from Case P. 5, fixed in Regaud's fluid and stained with Giemsa's solution, 14 days after removal from patient. The characteristic verrucous structure is still recognizable, although many cells have undergone degenera-

tion. The dark masses of irregular size are aggregates of minute bacilliiform organisms. $\times 152$.

FIG. 18. The same section as that shown in Fig. 17 but under a higher magnification. $\times 1,000$.

FIG. 19. Appearance of colonies of the verruga organisms in the upper portion of a tube of leptospira medium. 28 days old at 25°C. Natural size.

FIG. 20. Appearance of colonies of the verruga organisms on the surface of horse blood agar slant, 12 days old at 25°. Natural size.

FIG. 21. Dark-field view of the culture shown in Fig. 19. $\times 1,000$.

FIG. 22. Dark-field view of the culture shown in Fig. 20. $\times 1,000$.

FIG. 23. The verruga organisms from a blood agar slant culture, 11 days old at 25°C. Giemsa's stain. $\times 1,000$.

FIG. 24. Flagella of the verruga organisms. Zettnow's stain. $\times 2,000$



Intradermal inoculation, 30 days.



Scarification, 16 days.



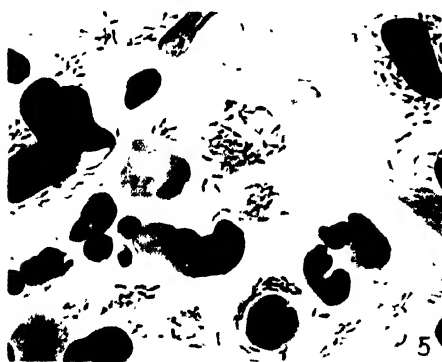
Blood, 30 days. Blood, 52 days.



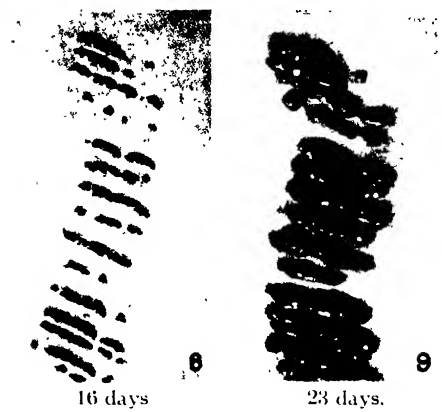
Scarification, 23 days



eyebrow nodule, 11 days



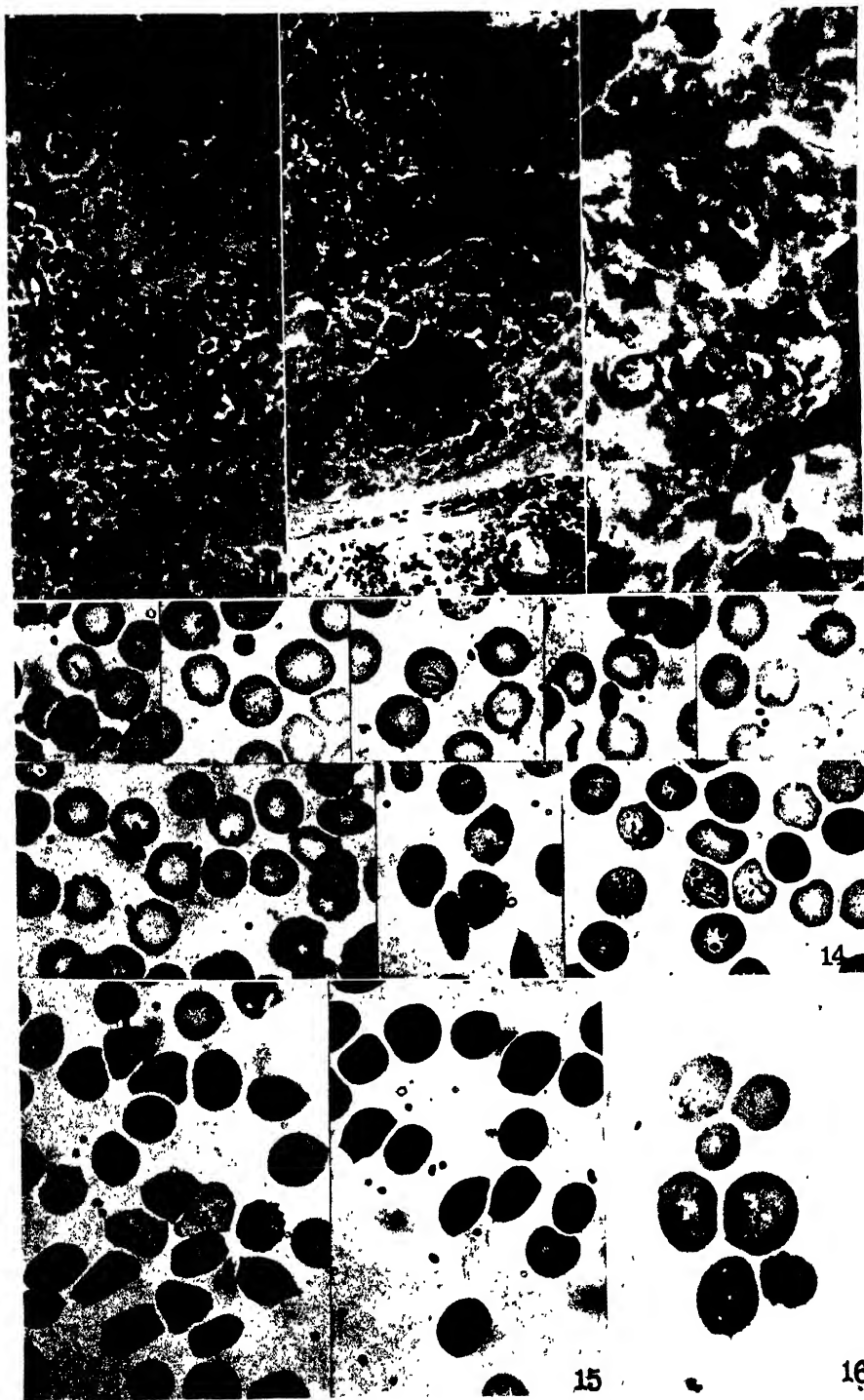
Human nodule.



16 days 23 days.



Nodule on tail.





(Noguchi: Etiology of verruga peruana.)

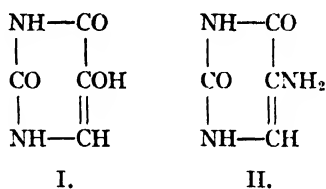
THE OXIDATION OF 5-AMINOURACIL.

BY OSKAR BAUDISCH AND DAVID DAVIDSON.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

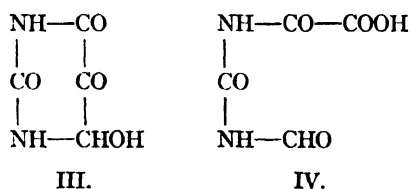
(Received for publication, November 16, 1926.)

The results obtained by the authors in the oxidation of isobarbituric acid I¹ by means of ferricyanide have led them to a study



of the behavior of this reagent toward other 5-substituted pyrimidines, of which 5-aminouracil II will be discussed in this paper.

Earlier investigators have shown that bromine water converts 5-aminouracil into isodialuric acid III² while permanganate splits



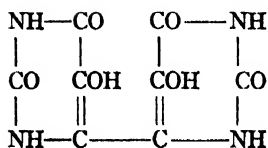
the ring producing formyl-oxalylurea IV.³ As in the case of isobarbituric acid¹ ferricyanide here too yields a unique oxidation product.

It was anticipated that just as the corresponding hydroxy compound (isobarbituric acid I) yields diisobarbituric acid V so 5-aminouracil II might yield 5,5'-diaminodiuracil. The reaction probably proceeds through this stage but analysis of the reaction product

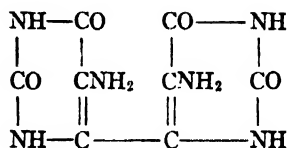
¹ Davidson, D., and Baudisch, O., *J. Biol. Chem.*, 1925, lxiv, 619.

² Behrend, R., and Roosen, O., *Ann. Chem.*, 1889, cccli, 235.

³ Offe, G., *Ann. Chem.*, 1907, cccliii, 278.

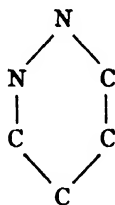


V.

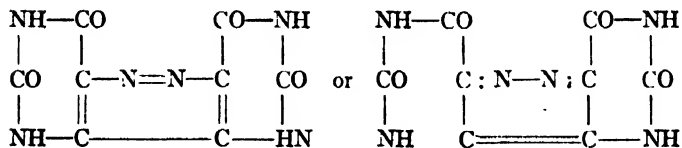


VI.

leads to the conclusion that oxidation continues further. If initial linking of the 4 carbons of two aminouracil rings be assumed, the ferricyanide could then act in its characteristic manner⁴ upon the two juxtaposed amino groups and thereby produce a double bond between the nitrogen atoms involved. Thus a third cycle—the pyridazine ring VII—would be formed and there would result the tricyclic diuracil pyridazine VIII.



VII.



VIII.

EXPERIMENTAL PART.

5-Aminouracil was prepared by the method of Johnson and Matsuo⁵ from 5-nitouracil.⁶

Diuracil Pyridazine.—A chilled, filtered solution of 38.8 gm. of potassium ferricyanide in 250 cc. of water was gradually added with mechanical stirring to a chilled, filtered solution of 8.9 gm. of potassium hydroxide and 5 gm. of 5-aminouracil in 150 cc. of water. Al-

⁴ Bamberger, E., and von Goldberger, A., *Ber. chem. Ges.*, 1898, xxxi, 2639.

⁵ Johnson, T. B., and Matsuo, I., *J. Am. Chem. Soc.*, 1919, xli, 782.

⁶ Davidson, D., and Baudisch, O., *J. Am. Chem. Soc.*, 1926, xlviii, 2379.

most at once precipitation of the brick-red potassium salt of diuracil pyridazine began. Complete precipitation of the product was secured by stirring for a few minutes after all the ferricyanide had been added. After passing in CO_2 to insure absence of alkali the reaction mixture was centrifugalized, the product washed by suspending in 1 per cent KCl and centrifugalizing again. After a second washing, the salt was suspended in alcohol, filtered, and washed with alcohol. In this manner, the product was conveniently obtained as a fine powder. From a dilute solution of the potassium salt, silver nitrate precipitates a deep wine-colored silver salt.

To obtain the free pyridazine, the potassium salt was suspended in water and poured into 200 cc. of hot water containing an excess of hydrochloric acid. The highly insoluble diuracil pyridazine separated immediately in orange-yellow diamond-shaped platelets. On cooling, the material was centrifugalized and then washed with water and alcohol as in the case of the potassium salt. Yield 2.5 gm. (51 per cent of theory).

Analysis.

$\text{C}_8\text{H}_4\text{O}_4\text{N}_6$.	Calculated.	C 38.69, H 1.63, N 33.88.
	Found.	" 38.74, " 1.84, " 33.66.

Diuracil pyridazine does not melt below 300° . It is insoluble in common organic solvents, very sparingly soluble in water, soluble in concentrated H_2SO_4 . When strongly heated in a tube it decomposes while a reddish yellow sublimate is produced, which dissolves in organic solvents and water to give strongly fluorescent solutions. It is unattacked by concentrated nitric acid or bromine water. With sodium hydroxide it at first forms the insoluble red sodium salt but dissolves in excess of alkali to form a yellow solution with beautiful orange fluorescence.

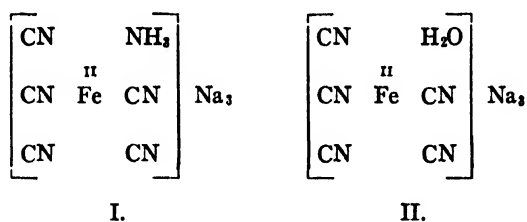
CATALYTIC OXIDATION BY MEANS OF COMPLEX IRON SALTS.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 16, 1926.)

The observation of Manchot¹ that sodium pentacyano-ammine-ferroate² I absorbs oxygen has led Baudisch and his coworkers³ to study the catalytic action of this salt, as well as of its water analogue II, in the oxidation of pyrimidines and purines. The present authors have previously reported in this journal⁴ on the nature of the oxidation products obtained. In the present paper the problem is considered from the standpoint of the catalyst. The work has been limited to sodium-pentacyano-ammine-ferroate I because of the ready availability of this salt in a satisfactory state of purity.



Theoretical.

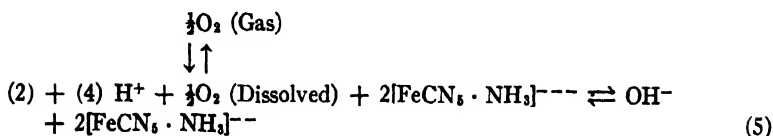
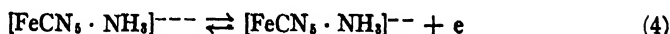
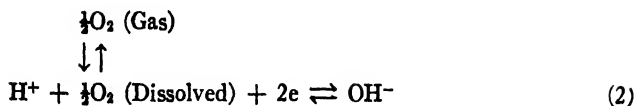
In the system: water, sodium pentacyano-ammine-ferroate, oxygen, organic molecule, the state of affairs may be represented by the following equations.

¹ Manchot, W., *Ber. chem. Ges.*, 1912, xlv, 369.

² Hofmann, K. A., *Ann. Chem.*, 1900, cccxii, 1.

³ Pfaltz, M. H., and Baudisch, O., *J. Am. Chem. Soc.*, 1923, xlv, 2972. Pfaltz, M. H., *J. Am. Chem. Soc.*, 1923, xlv, 2980. Baudisch, O., and Bass, L. W., *J. Am. Chem. Soc.*, 1924, xlvi, 184.

⁴ Baudisch, O., and Davidson, D., *J. Biol. Chem.*, 1925, lxiv, 233. Davidson, D., and Baudisch, O., *J. Biol. Chem.*, 1925, lxiv, 619.



Equation 2 represents the tendency of hydrogen ion to react with dissolved oxygen, taking up 2 electrons to form hydroxyl ion. Equation 4 indicates the tendency of the complex ferroate ion to give up 1 electron to become the ferriate ion, while Equation 5, which combines Equations 2 and 4, sums up the reactions involved. Though Equation 5 is actually not reversible yet it becomes so in the presence of an oxidizable organic compound such as a pyrimidine, which can act as an acceptor for the oxygen theoretically capable of being liberated. Since, moreover, the oxidation of the pyrimidine is certainly not reversible, the ferriate ion produced by the forward process of Equation 5 is continuously reduced to the ferroate ion by the pyrimidine present so that the complex ion, therefore, acts as an oxygen carrier or oxidation catalyst.⁵

Apparatus.

The course of the catalyzed oxidations represented in the accompanying charts was followed by measuring the oxygen absorbed. The Van Slyke amino apparatus⁶ was adapted to this purpose. At the beginning of an experiment the apparatus was completely filled with water. By adjusting the stop-cocks properly the excess of water in vessel *A* was allowed to run out. The leveling bulb was lowered and about 65 cc. of oxygen passed from a reservoir into burette *F* through

⁵ In this connection see Smith, J. H. C., and Spoehr, H. A., *J. Am. Chem. Soc.*, 1926, *xlvi*, 107.

⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1912, *xii*, 278.

stop-cock *c*, which was then turned, and the leveling bulb raised above the apparatus. The compressed gas in the burette was now allowed to force out through stop-cock *d* the water contained in the vessel *D*. By this means the water originally in the apparatus was displaced by oxygen. Lowering the leveling bulb until the gas in the apparatus was below atmospheric pressure allowed solutions or suspensions to be drawn into the deaminizer *D* from its burette *B*. The volume of *D* having previously been found, the total volume of gas in the apparatus at any moment during an experiment was determined by reading the level of the water in burette *F*.

In the experiments described the volume of solution used was 20 cc. A suspension of the organic compound in 10 cc. of buffer solution was first introduced, followed by 10 cc. of a solution of proper concentration of the iron salt, prepared just before use in an evacuated test-tube. The shaker was operated at 500 strokes per minute. The temperature was 20°C. ($\pm 2^\circ$) and the pressure 760 mm. (± 10 mm.). The volumes recorded in the figures are of moist oxygen reduced to standard conditions.

The buffer solutions employed were made up so that each 100 cc. contained: for pH 2, 9.8 gm. of H_3PO_4 + 9.1 gm. of KH_2PO_4 ; for pH 7, 7.1 gm. of Na_2HPO_4 + 4.5 gm. of KH_2PO_4 ; for pH 12, 9.5 gm. of Na_3PO_4 + 0.4 gm. of KH_2PO_4 .

DISCUSSION OF RESULTS.

As is seen from Equation 5 the oxidation of the pentacyano ammine-ferroate ion by elementary oxygen involves hydrogen ion. It is, therefore, to be expected that the rate of oxidation of the complex ion will be dependent on the hydrogen ion concentration. That this rate is actually inversely proportional to the pH of the solution is seen in Fig. 1.

It is also a consequence of Equation 5 that aside from other considerations the rate of reduction of the complex ferriate ion will be dependent on the hydroxyl ion concentration; that is to say, directly proportional to the pH. The ability of the pentacyano-ammine-ferroate ion to act as an oxygen carrier depends not only on the rate of oxidation of the complex ferroate ion by elementary oxygen, but

also on the rate of reduction (in the presence of some organic compound) of the complex ferriate ion (essentially the oxidation potential of the ferriate-ferroate mixture). Since these two rates are *oppositely*

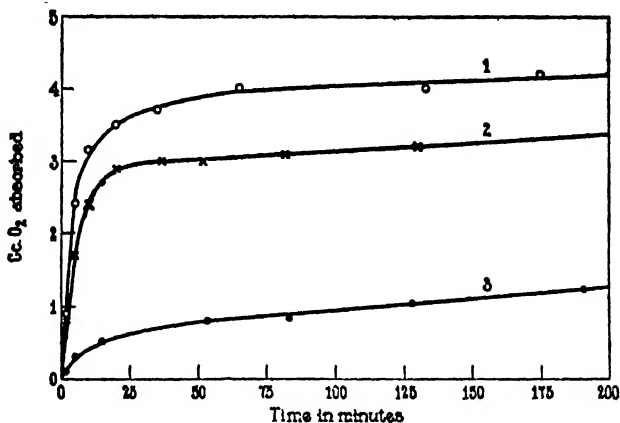


FIG. 1. The rate of oxygen absorption by the pentacyano-ammine-ferroate ion is inversely proportional to the pH of the solution.

In each experiment, 1 millimol of the complex iron salt was dissolved in 20 cc. of buffer solution. Curve 1, pH 2; Curve 2, pH 7; Curve 3, pH 12.

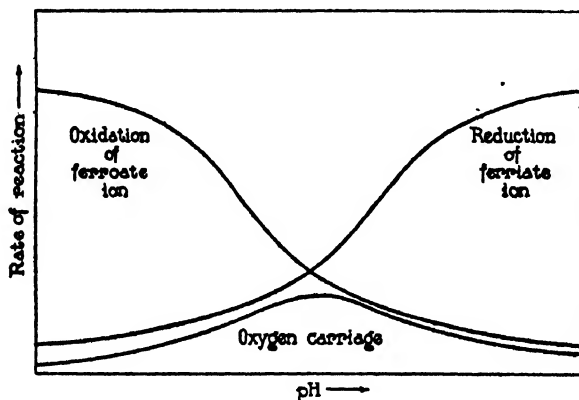


FIG. 2.

affected by the pH of the solution, it follows that what might be called the rate of oxygen carriage will have a *maximum* at some pH from which it will fall off on either side. These relations may be illustrated schematically as in Fig. 2.

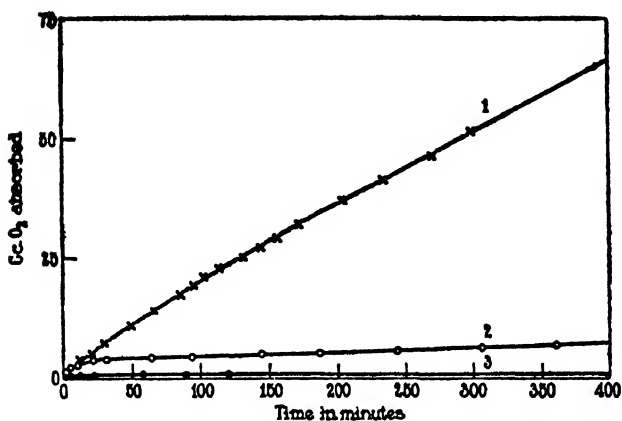


FIG. 3. The catalyzed oxidation of hydantoin is more rapid at pH 12 than at pH 7.

In each experiment, 10 millimols of hydantoin were suspended in 20 cc. of buffer solution containing 1 millimol of sodium pentacyano-ammine-ferroate. Curve 1, pH 12; Curve 2, pH 7; Curve 3, pH 12 (without catalyst).

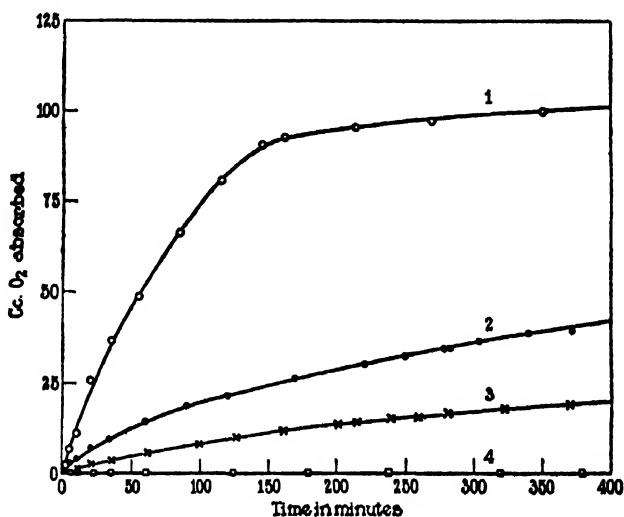


FIG. 4. The catalyzed oxidation of isobarbituric acid is more rapid at pH 12 than at pH 7.

In these experiments, 5 millimols of the pyrimidine were suspended in 20 cc. of buffer solution containing 1 millimol of catalyst. Curve 1, pH 1; Curve 2, pH 7; Curve 3, pH 12 (without catalyst); Curve 4, pH 7 (without catalyst).

Besides the variation of oxygen carriage power with pH must also be considered the change of oxidizability or the "apparent oxidation potential"⁷ of the organic compound. At all events the pH at which oxygen carriage to a given organic compound may be optimum will not necessarily be that at which the amount of oxygen absorbed by the pentacyano-ammine-ferroate ion itself is large as it is, for example, in acid solution, but may actually occur in the alkaline range where the oxygen absorption of the complex salt itself is relatively low. As a

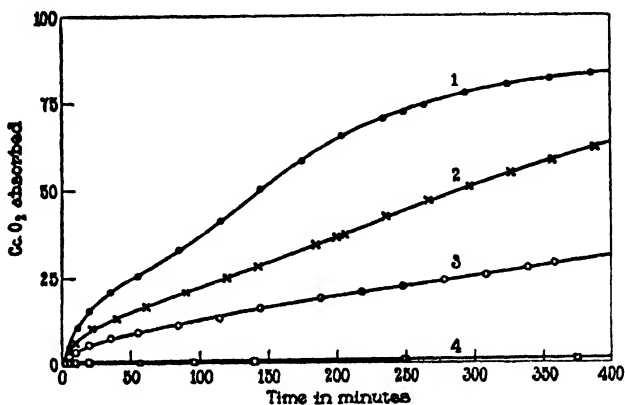
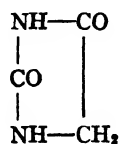


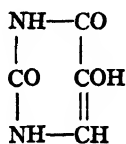
FIG. 5: The rate of catalytic oxidation of 5-aminouracil is proportional to the concentration of catalyst (pH 12).

In each experiment, 5 millimols of the pyrimidine were suspended in 20 cc. of buffer solution which contained a varying amount of complex salt as follows. Curve 1, 2 millimols; Curve 2, 1 millimol; Curve 3, $\frac{1}{2}$ millimol.

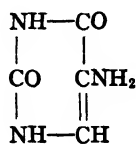
matter of fact in the investigated cases of hydantoin III⁸ (Fig. 3), isobarbituric acid IV⁹ (Fig. 4) and aminouracil V,¹⁰ the optimum is somewhere on the alkaline side, since the rate of catalyzed oxidation is greater at pH 12 than it is at pH 7.



III.



IV.



V.

⁷ Conant, J. B., *Chem. Rev.*, 1926, iii, 23.

⁸ Harries, C., and Weiss, M., *Ann. Chem.*, 1903, cccxxvii, 369.

⁹ Davidson, D., and Baudisch, O., *J. Biol. Chem.*, 1925, lxiv, 619.

¹⁰ Baudisch, O., and Davidson, D., *J. Biol. Chem.*, 1927, lxxi, 497.

From Equation 5 it may be seen that the rate of oxygen carriage will also be dependent on the concentration of the complex ion. This relation is amply illustrated in the case of the oxidation of 5-aminouracil represented in Fig. 5.

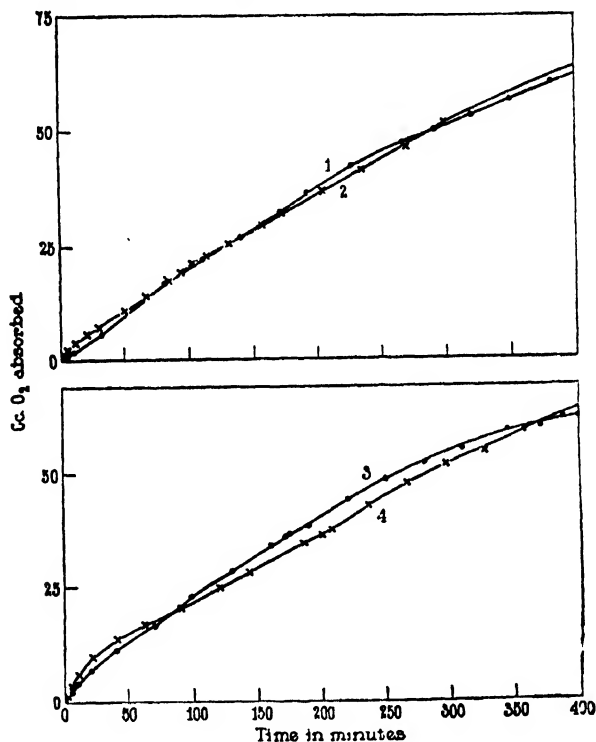


FIG. 6. Pentacyano-ammine-ferriate and ferroate are equally efficacious as oxygen carriers (pH 12).

Curve 1, 10 millimols of hydantoin with 1 millimol of ferrous salt. Curve 2, 10 millimols of hydantoin with 1 millimol of ferric salt. Curve 3, 5 millimols of 5-aminouracil with 1 millimol of ferrous salt. Curve 4, 5 millimols of 5-aminouracil with 1 millimol of ferric salt.

As previously stated Equation 5 is reversible only when an oxidizable organic compound is present to absorb the oxygen involved. This is borne out experimentally by the fact that when sodium pentacyano-ammine-ferriate is tested, as the corresponding ferrous salt previously was in pH 2, 7, and 12 (see Fig. 1) it *neither evolves nor absorbs oxygen*.

Since the cycle of reactions postulated above for the mechanism of

the oxygen carrier action of sodium pentacyano-ammine-ferroate involves, in one stage, the reduction of the complex ferriate ion (Equation 5), essentially the same catalytic action should be exhibited by the ferriate salt as by the ferroate salt. That this condition is fulfilled is demonstrated in Fig. 6 in the cases of hydantoin and 5-aminouracil.

SUMMARY.

1. Pentacyano-ammine-ferroate ion is oxidized by elementary oxygen at a rate which is inversely proportional to the pH of the solution.
2. In the cases studied the rate of catalyzed oxidation is greater in alkaline solution than in neutral.
3. In the cases studied pentacyano-ammine-ferroate and pentacyano-ammine-ferriate ions have identical catalytic activity.

ON THE OCCURRENCE OF MUTATIONS WITHIN TRANSPLANTABLE NEOPLASMS.*

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INTRODUCTION.

In a survey of the recent work on the inheritance of susceptibility to a transplantable tumor, two general conclusions of importance can be deduced. The first of these conclusions formulated by Little and Strong (1924) is that the fate of the implanted tumor tissue is determined by a reaction between the host and the transplantable tumor cells. The reaction of the host is governed largely by its genetic constitution (derived from the zygote from which it arose) and that of the transplantable tumor is somewhat controlled by certain intrinsic or genetic factors. The expression "reaction potential" perhaps defines this play of factors which determine the "transplantability" of a tumor more accurately than the older expressions "proliferative or growth vigor" and "adaptability." These older expressions overemphasize the rôle of the tumor transplant at the expense of the important part played by the host.

The second conclusion, concerning the host's tolerance of a transplantable tissue is more specific, "susceptibility to a transplantable

* The experiment here reported has been made possible by a grant from The Rockefeller Institute for Medical Research.

neoplastic tissue is brought about by the simultaneous presence of *multiple* Mendelian (genetic) factors, introduced into the individual at the time of the fertilization of the egg" (Little and Strong 1924). With the use of the term "simultaneous presence," we do not intend to imply any reference to the discarded theory of presence and absence first introduced by Bateson. We use the term "presence" for purely descriptive purposes. For example, if susceptibility to a transplantable tumor depended upon the simultaneous presence of two genetic factors, *A* and *B*, (as in the case of the adenocarcinoma, *dBrB*), then the mice with the following genetic constitutions would

TABLE 1.

Expected proportions of susceptible (+) and non-susceptible (−) individuals in the two important generations, the F₂ and the backcross to the negative strain, on the assumption that for the continued growth of the transplant there must be present one or more definite genetic factors within the host.

NUMBER OF FACTORS	FACTORIAL COMPOSITION F ₁	EXPECTATIONS F ₂	RATIO IN F ₂	PER CENT NEGATIVES F ₂	EXPECTATIONS, BACKCROSS
1	<i>Aa</i>	3+:1−	1+:0.33−	25.00	1+:1−
2	<i>AaBb</i>	9+:7−	1+:0.78−	43.75	1+:3−
3	<i>AaBbCc</i>	27+:37−	1+:1.37−	57.81	1+:7−
4	<i>AaBbCcDd</i>	81+:175−	1+:2.16−	68.38	1+:15−
5	<i>AaBbCcDdEe</i>	243+:781−	1+:3.21−	76.27	1+:31−
6	<i>AaBbCcDdEeFf</i>	729+:3367−	1+:4.61−	82.20	1+:63−
7	<i>AaBbCcDdEeFfGg</i>	2187+:14197−	1+:6.49−	86.65	1+:127−
8	<i>AaBbCcDdEeFfGgHh</i>	6561+:65536−	1+:9.98−	90.89	1+:255−

be susceptible; *AABB*, *AaBB*, *AABb* and *AaBb*. The mice with the following genetic constitutions would be resistant to the transplant; *AAbb*, *Aabb*, *aaBb* *aaBB* and *aabb*.

The Mendelian expectations for susceptible and non-susceptible individuals, involving the several possible combinations of "simultaneous factors," for the two generations, the F₂ and the backcross to the negative strain, are given in table 1.

Experimental Methods.

The tumor which is the subject of this investigation is the third spontaneous neoplasm to arise in a relatively homogeneous dilute

brown strain of mice which had been rigidly inbred, brother to sister, for at least forty generations. This tumor, which has not been previously described, we have designated as *dBrC*, in order to continue the nomenclature used for the other tumors arising in this same stock which were called *dBrA* and *dBrB*. On transplantation it was found, as with the previous tumors, that all animals of this dilute brown strain were uniformly susceptible. On the other hand, the Bagg albino strain, another inbred stock, was found to be uniformly resistant to transplants of this tumor as it had been to tumors A and B of the same stock.

TABLE 2.

Comparison of the observed data for the F₂ generation with the expectation for five, six and seven Mendelian factors, in case of original dBrC tumor.

F ₁ GENERA- TION	NUMBER OF INDIVIDUALS	PER CENT NEGATIVES	DIFFERENCE BETWEEN
1 Observa- tion	23.00+ : 102- ± 2.89	81.60 ± 2.30	
2 Expecta- tion 5 Factors	29.69+ : 95.31- ± 3.17	76.09 ± 2.69	1 and 2 = 5.51% ± 3.53 or 1.56 × P.E.
3 Expecta- tion 6 Factors	22.28+ : 102.72- ± 2.85	82.16 ± 2.30	1 and 3 = 0.56% ± 3.25 or 0.17 × P.E.
4 Expecta- tion 7 Factors	16.69+ : 108.31- ± 2.54	86.65 ± 2.03	1 and 4 = 5.05% ± 3.05 or 1.65 × P.E.

The F₁ generation produced by crossing these two stocks gave individuals all of which grew the transplant (180+ : 0-). At the present time, 125 individuals from the F₂ generation have been inoculated with this transplantable tumor, *dBrC*, and 23 of these grew the transplant, while 102 proved to be resistant. In attempting to fit the observed data to Mendelian principles, as has already been so successfully done in the case of four transplantable tumors (two by Tyzzer and Little and two by Little and Strong 1924), it was evident

that the behavior of the transplant placed it in the same class as the two other transplantable tumors derived from this same inbred dilute brown stock of mice. The results can be explained if the assumption be made that for the successful growth of the transplant there must be present within the host at least from five to seven independently inherited genetic units or factors (genes). It is probable that the number of genetic units involved is six. The degree of probability determined by the comparison of the probable errors between observation and expectation for five, six and seven factors, is greater in the case of six factors than it is for the other two possibilities, although the five and the seven factor interpretation is not definitely excluded by the present data.

The degree of significance between the observation on the original *dBrC* tumor and the expectation according to Mendelian principles is given in table 2.

After the collection of the above data, no further genetic studies being contemplated, the tumor was simply continued for some generations in the original dilute brown stock. In the course of this routine continuation of the tumor it was found that one of the transplants grew with remarkable rapidity. In sub-transplants from this particular tumor the rapidity of growth continued. Whereas the original *dBrC* tumor, growing in the susceptible homogeneous dilute brown mice, would rarely attain a weight of a gm. and a half to two gm. in from two to three months, this single mass has produced upon further transplantation growths which have reached from twenty-two to twenty-three gm. in from four to five weeks. Obviously there must have been a clear cut variation from the type reaction which had been investigated for over a year. Consequently this new tumor was called *dBrCX*, the *X* being attached to the original formula signifying some unknown characteristic of the new transplantable tumor. On further study it soon became apparent that the substrain had developed new tissue characters. *dBrCX* grew in all F_2 's, in all F_1 's, and in all individuals belonging to the original dilute brown mice, in all individuals belonging to several backcross generations toward the original non-susceptible stock, in all individuals belonging to the original non-susceptible stock, and in fact in all mice irrespective of their genetic relationships. It grows equally well in wild mice and

TABLE 3.
The data obtained for the three transplantable tumors, dBrCm, dBrCsp and dBrCX, in the F₂ generation, together with the comparable data for the original dBrC tumor.

NAME OF TUMOR	F ₂ GENERATION	NUMBER OF INDIVIDUALS	PER CENT NEGATIVES	DIFFERENCE BETWEEN
Original dBrC	1 Observation	23.00+ : 102.00- ±2.89	81.60% ±2.30	
dBrCm	5 Observation 6 Expectation 1 Factor 7 Expectation 2 Factors 8 Expectation 3 Factors	99.00+ : 65.00- ±4.17 123.00+ : 41.00- ±3.55 92.25+ : 71.75- ±4.23 69.20+ : 94.80- ±3.60	39.64% ±2.53 25.00% ±2.16 43.75% ±2.57 57.81% ±2.20	1 and 5=41.96% ±3.42 or 12.26×P.E. 5 and 6=14.64% ±3.32 or 4.41×P.E. 5 and 7= 4.11% ±3.60 or 1.14×P.E. 5 and 8=18.17% ±3.35 or 5.42×P.E.
dBrCsp	9 Observation 10 Expectation 1 Factor 11 Expectation 2 Factors	75.00+ : 23.00- ±2.79 73.50+ : 24.50- ±2.85 55.10+ : 42.90- ±3.27	23.47% ±2.86 25.00% ±2.90 43.75% ±3.36	1 and 9=58.13% ±3.67 or 15.84×P.E. 9 and 10= 1.53% ±4.07 or 0.37×P.E. 9 and 11=20.28% ±4.41 or 4.59×P.E.
dBrCX	12 Observation	243.00+ : 2.00- ±0.94	0.82% ±0.38	1 and 12=80.78% ±2.48 or 32.57×P.E.

mice belonging to a stock of mice imported from England several years ago and kept entirely distinct by brother-to-sister matings since their importation. From a state of high specificity, the transplantable tumor had become completely non-specific. Correlated with this loss of tissue specificity there was also obtained an increased growth or proliferative vigor. There was apparent a degree of significance of difference between the original *dBrC* and the new *dBrCX* tumor amounting to 32.57 times the probable error. From the time at which this distinct deviation from the normal growth rate of the transplantable tissue was observed we made careful observations with the possibility of detecting other deviations from the estimated normal growth rate. As a result of this procedure, two transplants were selected which seemed to show a difference. These have since received the names of *dBrCm* and *dBrCsp*. *dBrCm* will grow in a given host, provided there be apparent the simultaneous functioning of at least two independently segregating Mendelian units, that is, there is obtained a typical 9:7 ratio in the F_2 generation. The ratio of susceptible mice to non-susceptible mice in the F_2 generation for the *dBrCsp* tumor is probably 3:1. In other words, the physiological difference between susceptibility and non-susceptibility is apparently controlled by the functioning of one Mendelian dominant factor introduced into the individual with the formation of the zygote. The data obtained for the three transplantable tumors, *dBrCm*, *dBrCsp* and *dBrCX* in the F_2 generation together with the comparative data for the original *dBrC* tumor are given in table 3.

Results.

A transplantable tumor, known as *dBrC*, gave very uniform results in a series of mice of known pedigree for a period of about a year. We may say that the reaction potential existing between the tumor and the individuals inoculated was the same during this first part of the experiment. This tumor will grow in a mouse progressively provided there be present simultaneously at least from five to seven independently segregating genetic factors (genes) in the fundamental make-up of the host derived from the zygote that gave rise to the individual. In the course of routine transplantation of this tumor, there

was a sudden change in this hitherto constant reaction potential resulting in at least three types of transplantable tumors—new types as far as their physiological activity and reaction potential are concerned. With this change in the reaction potential of the transplantable tumor there is also involved or correlated with it a changed proliferative vigor and other physiological changes. From a single original transplantable mass, I have therefore obtained four masses that remain true to type at least for some months of continued experimentation. These four types are (1) the original tumor, *dBrC*, still giving a 5-7 Mendelian factor ratio, (2) *dBrCm* giving a two factor ratio, (3) *dBrCsp* showing a one factor ratio, and (4) *dBrCX* growing in all mice inoculated—that is non-specific. The number of factors involved in the “transplantability” for the several tumors investigated is given in table 4.

TABLE 4.

Tabulated synopsis of the various transplantable tumors used in the present experiment, showing the probable number of independently segregating genetic factors involved in the process of transplantation.

NAME OF TUMOR	NUMBER OF GENETIC FACTORS
Original <i>dBrC</i>	6
<i>dBrCm</i>	2
<i>dBrCsp</i>	1
<i>dBrCX</i>	Undetermined

GENERAL DISCUSSION.

Transplants from a spontaneous cancer rarely grow well in the first generations. Generally, however, the number of successful takes increases with subsequent transplantation till a very high rate is attained. There are two important theories formulated to explain this supposed increased ease of transplantation. The first theory may be called the virulence theory, although this term is rather unfortunate on account of certain implications from the study of infectious diseases. The second theory has been called the adaptation theory and is described by Woglom (1919) as “The presence (or absence) of power to adapt themselves to new hosts appears to be a

deciding factor in the success (or failure) of most spontaneous mouse carcinomata after transplantation." The first theory is fostered by Ehrlich and Apolant, the second by Bashford, Murray, Haaland, Bowen, Cramer and more recently by Woglom.

The data contained in this paper have a direct bearing upon these two theories. It is very probable that neither the theory of adaptation nor the theory of virulence will explain completely the evidence outlined. These theories are descriptive phrases applied to the tumor mass and enter into the equation after the change or changes have been produced—that is, they are the result of the change and not the cause of the phenomenon. As in the case of so many other alternative hypotheses, a third explanation is sometimes called for that seems to fit the data better than either of the original hypotheses.

Before attempting to formulate this new hypothesis, let us enumerate a few of the features involved in the transplantation of malignant tumors, that have a bearing on the problem at hand. (1) The cell is the unit of structure in the malignant tissue as well as it is in the normal tissue. (2) The inoculation of a given mass consisting of malignant tissue results in a true transplantation of tissue and is not an infectious process. By this is meant that the cells making up the mass of the new growth are lineal descendants from the cells introduced into the host at the time of inoculation. (3) Very few cells placed in the new host give rise to the new growth; most of them die during the process. (4) Uniformity of behavior of the transplantable tumor implies, of course, that there must be a relatively stable mechanism for the perpetuation of this uniformity.

With these conceptions concerning the process of the transplantation of malignant tissue before us, we are now ready to attempt an analysis of this phenomenon of the change in the reaction potential encountered in the present experiment. The change, whatever it is, must of course be either in the host component or in a possible tumor variable.

Obviously, if the host's tolerance of a transplantable tumor is dependent to a large extent at least upon its genetic constitution, when a change occurs in the genetic constitution of the host then this change, of necessity, would alter the reaction potential under con-

sideration. Changes in the genetic constitution of the mice, however, cannot explain the present data. The second filial generation individuals, which give the indication of the genetic complex involved so far as the host is concerned have been picked out at random and inoculated with one type of transplantable tumor or another. The parents were the same for all the F_2 's, while the number of susceptible grandparents was very small. Sometimes a single mouse was inoculated with two different types of tumors simultaneously. The genetic constitution of the mouse, as far as this experiment on testing its physiological response to two distinct types of tumors is concerned could not possibly be different. For these two reasons, the host complex during this experiment must have been a constant one or, at least, has not appreciably influenced the observed change in the reaction potential. The variable factor has not been within the mice employed.

We are therefore led to the conclusion that the only other variable that may be functioning is the transplantable tissue. There are two alternative conceptions concerning the mechanism of how this change in the tumor may have been brought about. (1) The original transplantable tumor employed was a mixed tumor, or (2) there has been a change in the genetic constitution of the unit of structure of the transplantable mass.

For several very obvious reasons, that need not be entered into here, the original tumor could not have been a "mixed" tumor.

The other conception is that this change of the reaction potential existing between the host inoculated and the tumor is brought about by an internal change in the genetic constitution of the tumor cell. This conclusion of course implies that the tumor cell is controlled to some extent at least by an internal genetic constitution.

Changes within the genetic constitution of living forms, if they are germ cells are called mutations; if they are somatic cells (occurring especially in plant tissues) are called somatic mutations. The conclusion is therefore reached that somatic mutations may occur within the tumor cell, and that when these mutations do occur, they change the reaction potential and other physiological activities of the tumor cell. The data at hand are not sufficient to determine more in detail the nature of this mutational process. It may be either a change or

shifting of a complete chromosome or chromosomes, or a change or changes within a chromosome or chromosomes (genic), or it may be even cytoplasmic in nature. By mutation, I merely mean to use the term in its broadest sense, that is, a change or shift within the genetic or internal constitution that results in definitely clear cut or discernible differences in behavior or structure that is perpetuated by the process of heredity (in this case, cell division).

We have therefore in the analysis of the data obtained in this experiment an explanation of the phenomenon of the so-called adaptiveness or virulence of the tumor cell. I have already pointed out the similarity between the data outlined in this paper and the data upon which the theories of adaptiveness and virulence or proliferative vigor are founded. If a genetic change such as a somatic mutation is responsible for the change in the reaction potential of the transplantable tumor cell and other physiological activities, such as the growth vigor then it is highly probable that the real cause of the phenomenon has been determined. A tumor increases its proliferative vigor or manifests an increased adaptiveness as a result of this mutational process. This somatic mutation may occur within a single cell in the neoplastic tissue. Then by the process of selective vitality or adaptiveness of the cells in the mass, the new type of behavior is obtained.

One other point I desire to mention. If one assumes that the spontaneous tumor possesses the same genetic constitution as the host that gave rise to it (and we have every reason to suppose that it does have, since the tumor has arisen by some process or other from cells derived from the body of the normal individual) then we must conclude from our present data, that a tumor mass may deviate from the genetic constitution of the host from which it arose, at least during the process of transplantation.

SUMMARY.

1. Mutations or hereditary genetic changes occur within the tumor mass at least during the process of transplantation.
2. These mutations result in a changed physiological activity of the tumor mass.
3. The theory of mutation explains the old phenomenon of the

increased percent of successfully growing masses on continued transplantation that has until the present been explained by the theories of adaptation and virulence.

4. The nature of this mutational process is as yet undetermined.

5. The tumor mass may deviate from the genetic constitution of the host that gave rise to it, at least during the process of transplantation.

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SEX AND THE CHROMOSOMES IN THE DOMESTIC FOWL (GALLUS DOMESTICUS).

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PLATES 1 TO 4.

AUTHOR'S ABSTRACT.

The chromosome number in the domestic fowl is approximately thirty-five or thirty-six. It is difficult to determine the exact number, owing to the smallness of the shortest chromosomes of the complex and also to the tendency of the chromomeres to occasionally appear as discrete chromosomes rather than as parts of a whole.

Difficulty experienced in fixing adult testes has prevented a satisfactory demonstration of all stages of spermatogenesis. However, satisfactorily preserved prophases of first spermatocytes have been observed which, together with the large amount of embryonic material available, have made it possible to work out the behavior of the sex-associated chromosomes with reasonable certainty. Measurements of the chromosomes indicate that the longest chromosome in the cell is single in the female and paired in the male. Two classes of eggs are therefore possible—one with and one without this long chromatic element. The female is therefore heterozygous and the male homozygous in regard to this chromosome which affords a cytological parallel for the genetic evidence of the heterozygosity of the female.

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¹ The major portion of this work was accomplished at the University of Pennsylvania during the writer's tenure of a National Research Council Fellowship.

INTRODUCTION.

The genetic heterozygosity of the avian female has long been recognized, but, owing to the difficulties of satisfactorily preserving bird tissues, an adequate cytological demonstration of this phenomenon has not been possible. About eight years ago, a generous contribution from Mrs. M. R. Lewis, of the Department of Embryology, Carnegie Institution of Washington, of beautifully prepared slides of chick tissue cultures interested the writer in the problems of bird chromosomes. Experimentation resulted in an excellent fixation of bird tissues and subsequently led to an understanding of the chromosome mechanism associated with sex in the chicken which proves to be in harmony with the available genetic evidence as well as with cytological data derived from other forms.

In order to profit directly by the experience of the principal student of avian chromosomes, M. F. Guyer, the manuscript and illustrations of this paper were sent to him. Doctor Guyer was good enough to review the paper and to send a letter expressing his opinion of the reported results in the light of his own work. The publication of this letter at the end of this article will, I feel, greatly aid the reader in forming an opinion of the present status of our knowledge of the mitotic phenomena of the domestic fowl, which is not likely to be classic cytological material as far, at least, as the chromosomes are concerned.

MATERIAL AND METHODS.

Fixation.

The experiments leading up to the successful technique have been described in full (Hance, '25). Living and killed tissue cultures have been very valuable in affording a standard to be equaled in the fixed samples of larger tissues. Strong Flemming's solution to which about 0.5 per cent urea had been added and used cold (about 0°C.) gave the best results. Adult testes and also chicken tumor tissue have proved to be very slowly penetrable by the fixative. The best preparations of testes obtained showed well-preserved mitotic figures only in the outer layer of tubules in direct contact with the fixing solution. Progressively poorer grades of preservation were found as one went nearer the center of the tissue, and this in spite of the

fact that the original sample of gonad was cut as small as could be done rapidly before immersing it in the preservative. This difficulty has recently been overcome in a study of tumors by first slicing as thin as possible with a razor and fixing these slices. It is hoped that similar treatment will result in a more uniform fixation of the adult testes of the fowl.

Materials.

The majority of tissue cultures studied were prepared in Baltimore by Mrs. M. R. Lewis. A few cultures were made by the writer. Embryos of from twenty-four to seventy-two hours' incubation, gonads from embryos incubated for from seven to nineteen days, and adult testes have been used in this study. In all, 184 specimens collected in Philadelphia, Pennsylvania, Fargo, North Dakota, and New York City have been examined. The collection and study of this material have extended over a period of about eight years.

The sexes of embryos may be readily differentiated at about the seventh day of incubation, when it is apparent in the case of the female that the left gonad is larger than the right. In male embryos the gonads are of equal size. The gonads from embryos of from fourteen to nineteen days' incubation usually contained a higher proportion of dividing cells than did those from younger chicks.

Publication of the complete results has been delayed for two years, owing to the difficulty experienced in obtaining adult testes, the cells of which were in an active state of division and well fixed. Even now the material in hand is not all that could be desired, but it is thought the excellence and number of the embryonic preparations compensated in some measure for this.

Drawings and Measurements.

The large amount of material available obviated the necessity of considering any but the clearest of cells. No cell was drawn unless every chromosome, with the possible exception of some of the smallest, stood out sharply. In general, few cells were selected for drawing that could not have equally well been photographed. This was important in view of the measurements that were made on the chromosomes. Unless the metaphase plate was perfectly flat, the full

length of the chromosomes would not be in one plane, and if this was not the case, measurements of them would mean little.

In all cases the chromosomes of each cell were drawn on a card, carefully checked with the original at the conclusion of the drawing, dated, and filed away. Later, the same cells were redrawn, checked, and then cross-checked with the first drawing. Occasionally as many as seven separate drawings were made of a single cell. This care was believed necessary to insure reasonable accuracy in the drawings which were eventually to be measured.

A small pair of dividers with points set 1 mm. apart was used in measuring the chromosomes as drawn. This method, while somewhat tedious, has proved to be more accurate and satisfactory than a number of other possible methods. The measuring was done on the final drawing referred to above and the dimension of each chromosome was printed near it on the card. The chromosomes of each group were traced in linear series according to size in a separate sheet (as in figs. 24 to 27). So little difference in length (however constant this may be) exists between chromosomes that if measurement of them had not been resorted to the conclusions of this paper would have been reached with difficulty, if at all.

OBSERVATIONS.

The Chromosome Number.

The exact number of chromosomes in the domestic fowl has been difficult and probably is impossible to determine with absolute accuracy for two reasons: First, the extreme smallness of the shortest chromosomes places them almost at the limit of microscopic vision, and this in many cases may interfere with the accuracy of the count. Secondly, and this is probably the more important factor, there is a tendency for the chromomeres of the smaller chromosomes to be either actually or apparently disconnected during the earlier stages of mitosis. The degree in which this pseudofragmentation exists, of course, varies the count considerably. The high counts are found only in the prophase stages, and as mitosis nears metaphase the number of individual chromatin particles becomes rapidly less, the total number becoming presumably fixed just before the chromosomes

are ready to divide. I have no reason to believe that these individual chromatin bodies which eventually must join to bring about the smaller number of chromosomes found at metaphase are actually disconnected in the first place. It would be rather strange if they were. It is more probable that they are linked by strands of linin which is invisible, but serves in the end to draw the proper granules together. Such invisible connections are suggested by the alignment of certain of the chromatin bodies in figs. 1, 2, 3, 29, and 30. Occasionally the connections are visible and have been illustrated in figs. 2, 3, 4, 11, 12, and 30. Though the majority of the prophase counts have been made from cells grown in tissue cultures as they are there found with unusual flatness and large size, their entire similarity to comparable stages of division in sectioned embryos has been fully established (Hance, '26).

With the above factors contributing to the numerical uncertainty in mind, it can be appreciated that, as far as the total number of chromosomes in the chick are concerned, we must resort to averages rather than to definite figures. The prophase counts vary from 40 to as high as 71 (fig. 1) depending on the nearness to metaphase—the number tending to reduce as mitosis proceeds. In cases where mitosis had apparently reached a true metaphase condition, the number ranged from 29 to 42, the average for seventy-eight cells being 33. My opinion, based on the clearest cells under observation, is that the actual count is nearer 35 or 36. Counts of the chromosomes in the first spermatocytes tend to bear out this belief as the average number there was found to be 17 with a range from 15 to 18. This average multiplied by 2 would be more than the average of the counts on diploid cells and 1 or 2 less than the estimated true number given above.

This difficulty in determining the chromosome number is caused apparently wholly by the smallest chromosomes of the series as the largest ones are ever present and constant in morphology. As already pointed out, this is due in part to the extreme smallness of these bodies as well as to the state of the union of the chromatin granules when the cell was fixed. The counts reported by other workers are considered in the discussion.

There can be no doubt, as suggested by Guyer ('16), that the

small bodies found in the center of the metaphase complexes are chromosomes. They fall in very well with the expected size series (discussed under section on chromosome measurements) and behave as chromosomes in staining reaction and at division. It is somewhat difficult to get an accurate count of these tiny bodies in anaphase, but I have at least one cell sufficiently good to permit of a count of the chromosomes at the two poles. Though the entire number cannot be made out, there are enough distinguishable to indicate the probable presence of the others. In fig. 6 the two poles of an anaphase with twenty-nine chromosomes in the left-hand drawing and twenty-six in the one on the right.

Unfortunately for the completeness of this account, a satisfactory enumeration of the spermatogonial chromosomes has not been made. This, as already stated, has been due to the mitotically inactive condition of the majority of adult testes that were studied and also to the difficulties of getting adequate fixation. It is hoped later to fill this gap in the record.

Chromosome Form.

There are but two characteristic shapes of chromosomes found in the chick cells, J's and rods. The larger chromosomes are all in the form of J's, while the shorter members of the complex are rods. At first sight, V's seem also to occur, but when averages are again resorted to it is found that the place in the length series where the occasional V naturally falls is filled with a preponderance of J's. One is faced with the conclusion that the few V's found are due to one, and perhaps two, of three causes: 1) they are actual variants from the normal; 2) they had not assumed final shape at the time of the fixation of the cell, or, 3) one arm may have been sufficiently foreshortened under the microscope to make it appear of similar length to the shorter arm. While in view of the work on heteromorphic chromosomes in grasshoppers the first possibility is not unreasonable, considering that the V condition is not constant in the same animal and in any case that the chromosomes are so small as to frequently make observation difficult, the second and third suggestions seem more likely.

The average condition, which I think is the usual one, found in the

chick complexes is that the first or longest six pairs of chromosomes are J-like in form and that all the others are rods. This has been diagrammatically presented in text fig. A. While these findings were made on embryonic cells, there is no reason to believe, on the basis of our knowledge of other forms, that the adult condition differs in any significant degree.

Although but few well-preserved spermatogonia have been studied, those observed show the longest pair to be J's just as in the male embryonic cells.

Chromosome Measurements.

It is obvious that the chromosome number, since it could not be definitely ascertained, would afford no aid in determining the chromosome sex mechanism. Consequently, it was necessary to resort to a study of the size relations of the various chromosomes of the complex in an attempt to find the chromosomes, if such there were, associated with sex. The chromosomes of seventy cells were measured and the chromosomes then arranged in a linear series according to length as illustrated in figs. 24 to 27. With all the care taken to insure accuracy of measurement as described under 'Methods,' it is questionable whether more than about half of the series are sufficiently large to permit of exact drawing. These longer chromosomes have, I am confident, been quite exactly outlined and the measurements on them are satisfactory. The lengths given for shorter members of the series are approximately correct, and, though they may serve for comparison, it would scarcely be wise to base on them any sweeping conclusions. Fortunately, as will be shown later, only the longest chromosomes of the complex are concerned with this particular problem.

Sample cells are illustrated in figs. 24 to 27 and the length in millimeters of each chromosome as originally drawn is placed alongside of the individual concerned. It is clear from an inspection of these figures that the total lengths of all the chromosomes of the various cells differ considerably, but that the relations between the pairs are essentially the same in the different cells as has been reported for other forms (Hance, '17, '18). Unless all of the difficulties involved in an attempt to accurately draw bird chromosomes are

held in mind, occasional variations in length between members of pairs when only a few cells are presented will occur that will perplex the reader. When large numbers are studied and their measurements averaged, many of these irregularities tend to disappear. When average lengths are considered, where the two members of the pair are not of identical proportions, they are much more nearly like each other than they are like the chromosomes on either side of them in the length series. It is also evident that, in general, the longer the chromosomes, the more nearly do the mates as drawn agree in length. The actual dimensions of the longer chromosomes may be conveniently considered in their relation to ascertaining the following.

TABLE 1.

	Pair number											
	1		2		3		4		5		6	
Chromosome number	1	2	3	4	5	6	7	8	9	10	11	12
Female gonad.....		20	17	15	13.5	12	11	11	9	9	8	8
Female-like embryo...		19	16	15	12.5	11	10	9	8	8	7	6
Male gonad.....	18.5	18	15	13	11.5	11	8.5	8.5	8	8		
Male-like embryo.....	17	17	14	12	10	9	8	7	6	6		

The Chromosomes Associated with Sex.

A study of the dimensions of the chromosomes in embryonic male and female gonads shows that in the female the longest chromosome is unpaired (figs. 7 to 9), while in the male the corresponding element has a mate (figs. 10 to 15). Furthermore, embryos of unknown sex fall into two classes, depending upon whether the longest chromosome is or is not paired. This may best be appreciated when the average lengths of ten or eleven long chromosomes of cells of embryonic male and female gonads and the two classes of embryos are presented. These figures are based on the averaged measurements of the chromosomes in seventy cells. In the following table and in the remainder of the article the terms 'male- or female-like embryo' refer to embryos whose chromosomal make-up is similar to that found in the male or female gonads.

Table 1 clearly indicates that in cells found in male gonads and

male-like embryos the two longest chromosomes are homologous and therefore, without doubt, members of the same pair. In the case of cells in female gonads and female-like embryos the longest chromosome is without a homologue. The other chromosomes of the series are obviously correctly mated.

This, then, is satisfactory evidence that the female is heterozygous for the longest chromosome, while the male is homozygous.

It is of interest, since earlier work (Hance, '17, '18) demonstrated the practical constancy of the inter-pair length relations, to examine the figures in table 1 for this condition. It is probably too much to expect in this instance quite the same constancy of results as was obtained with the more favorable material previously studied, but even so the percentage difference in length between the various chro-

TABLE 2.

	Pair numbers					Average
	1-2	2-3	3-4	4-5	5-6	
Female gonad.....	20	20	14	18	11	17
Female-like embryo.....	19	24	20	16	19	20
Male gonad.....	24	20	25	6		19
Male-like embryo.....	24	27	21	20		23
General average.....						20

mosome pairs in the chick prove to be quite similar—sufficiently close, I feel, to justify the belief that the chromosome length relations in this bird are similar to those existing in non-avian forms. Table 2 gives for the six longest pairs of chromosomes the percentage length differences between the pairs. In computing these differences when the length of the homologues differed slightly, the average length of the two members was used.

Comparing the general average length difference between pairs with the individual differences, it can be seen that there is, with two or possibly three exceptions, remarkably little deviation from the average or, probably we may say, constant value. This comparative similarity of the interpair relationships affords a further check on the accuracy of observations based on length alone. In other

words, the single long chromosome heading the female series differs in length from the next pair by approximately the same amount as does the first pair in the male series from the second pair. This would seem satisfactory proof of the accuracy of these deductions.

The spermatogonium pictured in fig. 16 shows the two large J-like chromosomes characteristic of the cells found in the embryonic male gonads.

The Spermatocytes.

The same difficulty was experienced in finding well-fixed spermatocytes as spermatogonia. Recently, a number of first spermatocyte prophases have been found that were located in the outermost tubule of the piece of testes as fixed and consequently, having come into almost direct contact with the fluid, are at least well enough preserved for reliable enumeration. I do not think that the fixation is at all delicate, as the tetrads exhibit none of the characteristic and familiar features found in most favorable material.

The number of chromosomes in the first spermatocyte prophases ranges from fifteen to eighteen, with an average of seventeen. The mere bulk of the chromosomes indicates their tetrad composition, and there can be little question that the largest one, usually of J-like form (see the chromosomes colored black in figs. 17, 18, 20, 21, 22, 23), has resulted from the synapsis of the two large J's found in embryonic male gonads, male-like embryos, and spermatogonia.

The second spermatocytes are in all cases poorly preserved, with the individual chromosomes fused together in an indistinguishable mass. It was useless to attempt to analyze them. They appear quite like those described by Guyer ('16), but I think, in view of the improvement effected in the fixation of the somatic chromosomes since his earlier investigations, that these cells have simply proved more resistant than the other and younger cells to the methods used and the conditions observed are solely artefacts. It is hoped that either a happy accident of fixation or an actually improved technique as suggested under 'Methods' may eventually aid in clearing up this phase of the problem.

DISCUSSION.

The chromosomes in the domestic fowl seem likely to remain a matter of controversy owing to their smallness and to their prophase habit simulating fragmentation. There can be no doubt, however, that the number lies somewhere in the thirties, my estimate placing it at thirty-five or thirty-six. Guyer reported ('16) eighteen as the most frequent number he observed, but also stated that if the small bodies in the center of the complex were chromosomes, the number might be as high as forty or fifty. It has been shown in this paper that the bodies are unquestionably chromosomes and should be included in the count. Miss Boring ('23), reporting some of Miss Stevens' notes and drawings of chicken chromosomes, indicates that the latter had rather better-preserved material than did some of the subsequent workers. Her spermatogonial figure (fig. 15 of this paper) was fairly clear and showed thirty-four chromosomes. This lies in between my average count of thirty-three and my estimate of thirty-five or thirty-six. Her first spermatocyte drawings (fig. 19, this paper), while similar in general appearance to mine (figs. 17, 18, 20, 21, 22, 23), show more chromosomes, the counts ranging from eighteen to twenty-two (average twenty), whereas mine vary from fifteen to eighteen, with an average of seventeen. The greater bulk of her chromosomes as illustrated may possibly be due to the fact that Miss Stevens relied to a considerable extent on the aceto-carmin method of fixing and staining which tends to cause the chromosomes to swell. A paragraph in an account of the work of the Moscow Institute (Koltzoff, '24) states that the number of chromosomes in the fowl is thirty-two, as established by Doctor Shivago.

An interesting similarity exists between reptilian chromosomes as portrayed by Painter ('19) and bird chromosomes both as to number and form. For the various species of reptiles described, the number of chromosomes ranges around thirty. The larger chromosomes are V- or possibly J-shaped. There is a much sharper distinction between the size of the larger and of the smaller chromosomes of the reptiles as drawn by Painter than has been found in the birds, where all formed a rather evenly graded length series.

The various number of chromosomes found by Miss Stevens,

Guyer, and myself in the spermatocytes is a matter deserving considerable attention. I feel reasonably certain, in view of the constancy of the behavior of cells in a vast number of other animals and plants, that there is not likely to be any irregularity in the behavior of the process of maturation. The variation in the chromosome number found in the diploid cells has been satisfactorily accounted for. The same explanation would not seem valid for the spermatocytes or, if ultimately it should prove to be true, it would be a most unique and interesting phenomenon. In my own work the number of observed first spermatocyte cells in division has been too few to serve as a basis of serious opinion. In the first place, the fixation of these cells is not perfect, which may have caused the clumping together of two or more chromosomes which would reduce the total number. The cells may have been cut and a few of the chromosomes either dragged out of the cell or located in another section. Or one or more chromosomes may have been hidden by the larger ones. All these factors contribute to the uncertainty of the counts of the chromosomes of the first spermatocytes. Although, of course, it has not been conclusively proved, it seems likely, in view of what has just been written, that the highest number (i.e., eighteen) is probably the one most nearly correct. It would be very unwise as yet, however, to completely lose sight of the possibility of there being an actual variation in the chromosome number of the spermatocytes, curious though it would be, and indeed, for that matter, in the diploid cells, though in the latter our evidence is to the contrary. Fragmentation of the chromosomes in the soma of some forms has already been demonstrated (Hance, '17, '18; Guyer, '16, also suggests this possibility for the chick), and though the conditions in the soma of the chick seem to be different, something of the same sort may also be concerned. In the other forms it will be remembered that the fragmentation of chromosomes was not observed in the cells of the germ line.

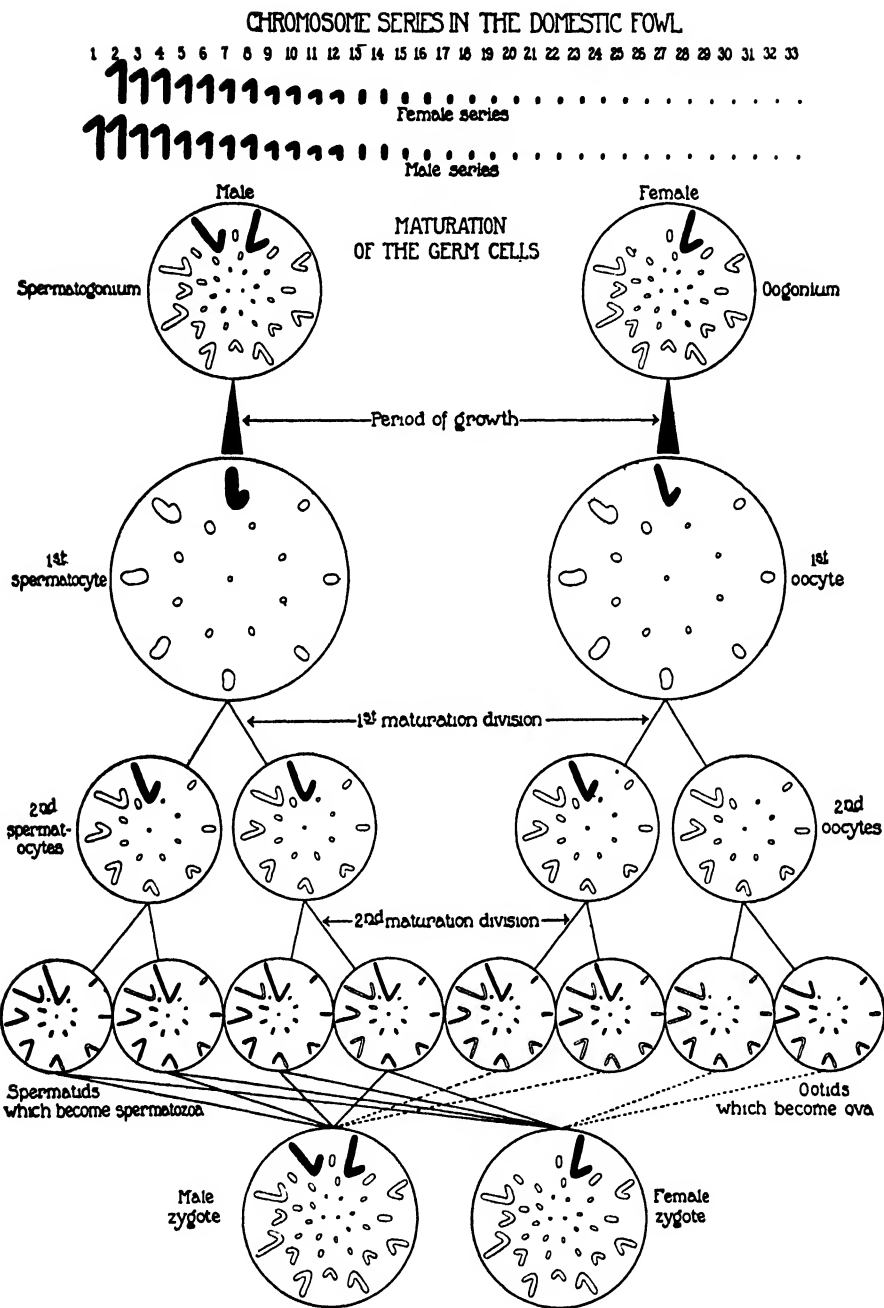
Since the chromosome number is not to be relied on for the determination of sex differences, it is fortunate that the measurements of the chromosomes have yielded the desired information. The problem would also have been unsolvable had the sex chromosomes not been the longest members of the series, as the accuracy of the measure-

ments naturally decreased with the size of the chromosomes. That the sex-associated chromosomes happen to be the longest ones in the cell makes their recognition doubly trustworthy.

This method of determining the sex chromosomes does not, of course, exclude the possibility of the presence of a Y-chromosome in the female. This is, however, a minor matter, and the principle of maturation and the sex behavior of the germ cells would not be affected in any known manner by its possible presence or absence.

For the better visualization of the processes of the maturation of the germ cells, the diagram in text fig. A was designed. Although many of the stages there illustrated have not actually been seen, I believe the phenomena proceed essentially as shown. The diagrammatic chromosomes are drawn to scale with a length interval of 20 per cent, as determined for the actual chromosomes, between pairs. Also for diagrammatic purposes the average chromosome number of thirty-three has been accepted. As the female is heterozygous, producing two classes of eggs, for simplicity of diagramming and for the sake of showing the two possible types of second oocytes, the polar bodies as such have been omitted. To gain a mental picture of what actually happens at this point one may imagine either of the second oocytes to be the polar body of the other and the chromosome mechanism illustrated will be correct. The manner in which male and female zygotes are produced is obvious from this chart.

I have only negative observations to place against Guyer's description of the non-division of the large chromosome in the first maturation division and these made on material but poorly fixed. Certainly, Guyer's figures clearly agree with his description regarding this and later conditions, but the poor preservation of the other chromosomes raises the question of the normality of the mitoses illustrated. Where chromosomes have been clumped together through improper fixation, it seems entirely possible that certain ones may be squeezed out of the main mass and stand alone, and it is well known that such fixation may produce all manner of variations in the apparent number of chromosomes. I have never seen anything to suggest this behavior, although I can hardly pretend that my experience is at all conclusive. This large chromosome, obviously and by Guyer's description, is a tetrad formed by the synapsis of the two large J's seen in the diploid



Text-fig. A. Diagram of the behavior of the chromosomes of the domestic fowl during the maturation of the germ cells. The description of the processes involved may be found in the text.

division figures. If it should fail to divide in the first division, it would be contrary to the usual behavior of tetrad chromosomes in other forms and comparable in a sense to non-disjunction, since it is placing into one cell double the amount of chromatic material (and presumably of genes) of any of the other chromosomes in the complex. While this in itself would seem to be a source of potential difficulties, the necessity of accounting for the class of second spermatocytes that fail to get their portion of this chromosome is even more troublesome. This phase of Guyer's presentation, together with his description of the occurrence of a secondary or pseudoreduction of the chromosome number in the second spermatocytes, may, I think, justly be attributed to the artefacts produced in tissues that are very difficult to fix properly. With Guyer's fundamental observation that sex is associated with the largest curved chromosomes in the cell I am in entire agreement, and I think this study has reasonably established the fact. Guyer's careful measurements of mature spermatozoa indicate beyond question that but one type is produced as determined by the length of head (this being an index of the amount of chromatin present), and this again agrees with the present findings, as all classes of spermatids receive the same complement of chromosomes.

These investigations make another interesting link in the chain of evidence that associates more and more closely the chromosomes, sex, and the mechanism of heredity.

LETTER FROM M. F. GUYER.

Madison, Wisconsin, July 7, 1926.

My dear Doctor Hance:

I have given your very careful study of the chromosomes in the domestic fowl a close inspection and find nothing in it with which to disagree. I congratulate you upon the skill and accuracy manifested in your results and I am sure they represent the most reliable observations yet made on fowl chromosomes. When last I worked on such material some eight years ago, I made use of tissue-cultures and chick amniotic membranes so that there could be no question of having cut away part of the cell, and I came to the conclusion that the full somatic number of chromosomes could not be less than 28 and that it was probably more if one counted all the deeply stained particles visible in the equatorial plate. The only doubt that I had was that probably the chromosomes seen in my tissue

cultures might not represent a natural state because the cells in such cultures were of abnormally large size. As to the passing over undivided of the fused sex chromosomes in the primary spermatocyte, I recorded only what I saw. I do not recall having ever found a single case that I could satisfactorily interpret as a division, although division is certainly what one would expect.

I met with the same difficulties you did in securing good fixation of spermatocytes in sections and that is why I resorted so freely to smears. However, it is obvious that smears might lead to distortions of the cell elements more easily than would carefully cut sections. I am not yet convinced that the secondary fusion of chromosomes in the secondary spermatocytes is an artefact due to fixation. In the first place, if I am not mistaken, I have seen this phenomenon a few times in presumably living cells in tissue cultures, and then again I recorded it as occurring in pigeons in my 1900 paper. The observation on pigeons was corroborated by Geoffry Smith in England some years later (1912).

Sincerely yours,

[Signed] M. F. GUYER,
Professor of Zoology.

SUMMARY.

1. The chromosome number in the domestic fowl is approximately thirty-five or thirty-six, it being difficult to determine the exact number, owing to the smallness of the shortest chromosomes of the complex and also to the tendency of the chromomeres to occasionally appear as discrete chromosomes rather than as parts of a whole.

2. Measurements of the chromosomes indicate that the longest chromosome in the cell is single in the female and paired in the male.

3. Two classes of eggs are therefore possible—one with and one without this long chromosome, while all the spermatozoa produced are alike in possessing the long chromatic element.

4. The female is therefore heterozygous and the male homozygous in regard to this chromosome which affords a cytological parallel for the genetic evidence of the heterozygosity of the female.

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EXPLANATION OF PLATES.

PLATE 1.

All drawings were made at table level with a Zeiss 1.5-mm. apochromatic objective and a 15 × orthoscopic ocular (Spencer). They are reproduced, with the exception of plate 3, at the size originally drawn.

1 Prophase chromosomes in cell growing in tissue culture. Seventy-one chromosomes or portions of chromosomes present. Compare with photomicrograph, fig. 29.

2 Prophase chromosomes in a tissue-culture cell. Nucleolus is just beginning to lose its power of stain retention. Fifty chromosomes present. Note the fine lines connecting certain chromosomes. See fig. 30.

3 Fifty-six chromosomes. Same comments as for fig. 2.

4 Chromosomes in tissue-culture cell nearing metaphase. Twenty-eight chromosomes.

5 Similar description to fig. 4. Thirty-eight to forty chromosomes.

6 Two poles of an anaphase. Twenty-nine chromosomes visible in the left and twenty-six in the right-hand figures.

PLATE 2.

Sex-associated chromosomes are drawn in solid black in all figures.

7 and 8 Metaphase chromosomes in cells of a female gonad. Fig. 7, thirty-four chromosomes; fig. 8, forty chromosomes.

9 Chromosomes from cell of a female-like embryo. Thirty-four chromosomes.

10 to 12 Metaphase chromosomes found in cells of male gonads. Fig.

10, thirty-five chromosomes; fig. 11, thirty-five chromosomes; fig. 12, thirty-nine chromosomes.

13 and 14 Chromosomes from male-like embryos. Fig. 13, thirty-three chromosomes; fig. 14, thirty-two chromosomes.

15 A copy of a drawing of spermatogonial chromosomes by N. M. Stevens, thirty-four chromosomes.

16 Spermatogonial metaphase, showing the two large J-shaped chromosomes. Central chromosomes not clear.

17 to 23 Drawings of first spermatocyte prophase chromosomes. Fig. 17, sixteen chromosomes; fig. 18, eighteen chromosomes; fig. 19, drawing by N. M. Stevens showing twenty chromosomes; fig. 20, sixteen chromosomes; fig. 21, seventeen chromosomes; fig. 22, eighteen chromosomes; fig. 23, fifteen chromosomes.

PLATE 3.

The chromosomes in these figures are shown both in their normal relations and arranged in series according to length. The numbers to the right of each chromosome of the various series indicate their length in millimeters as determined from the original drawings. Note that the longest chromosome in the female cells is unmated and paired in the male cells.

24 Chromosomes found in an embryonic female gonad. Twenty-nine chromosomes.

25 Chromosomes in the brain cell of a female-like embryo. Thirty-five chromosomes.

26 Metaphase plate in an embryonic male gonad. Thirty-nine chromosomes.

27 Somatic chromosomes in a male-like embryo. Thirty-two chromosomes.

PLATE 4.

Photomicrographs of mitoses in chick cells reproduced at a magnification of approximately $\times 1000$.

28 Late prophase from tissue-culture preparations. See fig. 5.

29 Early prophase from tissue culture. See fig. 1.

30 Early prophase from tissue culture. See fig. 2.

31 Polar view of metaphase in a dividing connective-tissue cell.

32 Metaphase cell in the neural tube of a chick embryo.

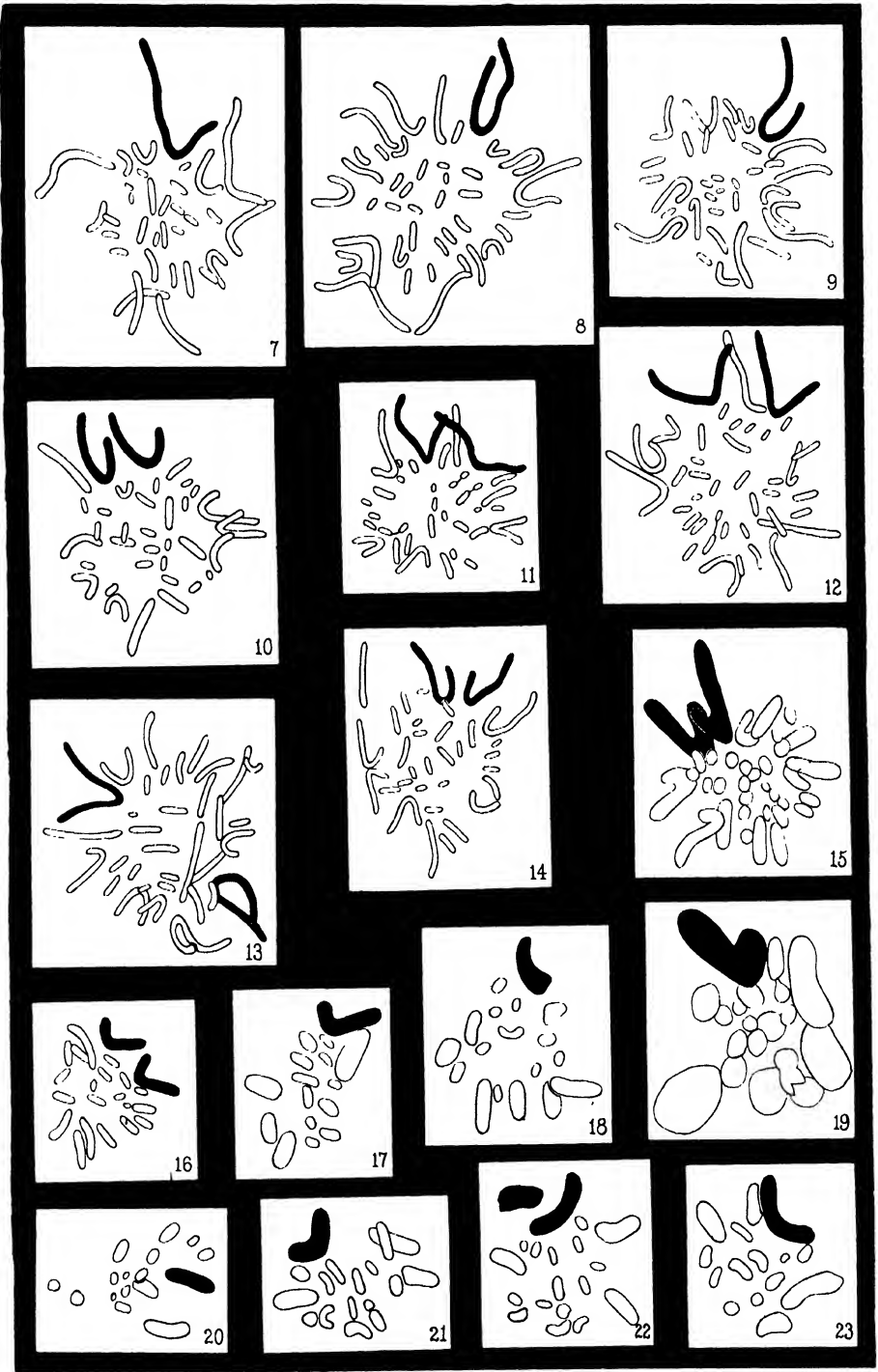
33 Metaphase found in a cell of an embryonic female gonad.

34 Same as fig. 32.

35 Metaphase from brain cell of chick embryo.



(Hance: Sex and chromosomes in domestic fowl.)



(Hance: Sex and chromosomes in domestic fowl.)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

(Hance: Sex and chromosomes in domestic fowl)



(Hance: Sex and chromosomes in domestic fowl.)

THE CHROMOSOMES OF THE CHICK SOMA.

By ROBERT T. HANCE.

(*From the Laboratories of The Rockefeller Institute for Medical Research and the Zoölogical Laboratory, University of Pennsylvania.*¹)

PLATE 1.

The accumulated evidence in favor of the chromosomes being in some way concerned with the determination and control of the somatic characters has become so impressive as to be almost unassailable despite the possible objection of a few that the last link in the chain (a demonstration of the actual activity of these nuclear bodies as hereditary character bearers), due to the deficiencies in our knowledge of cell chemistry, has yet to be forged. This latter point is one about which geneticists and cytologists are, at present, not greatly concerned and conclusive data upon the physiological behavior of the chromosomes does not seem at all imminent. While information is gradually accumulating in preparation for this final analysis much may still be accomplished by purely morphological studies with tentative interpretations of physiological activity based on these observations. A recent study of the chromosomes associated with sex in the chick (5) involved extensive observations on the chromosomes of dividing somatic cells, the results of which are recorded below.

OBSERVATIONS.

The technique of preparation (3), of counting and measuring the chromosomes, the amount and source of the material (5) have all been recorded and need not be repeated here. The chromosomes of over 150 cells have been drawn and studied. As the nervous system is the most actively growing of all the regions of the body the majority of the cells studied have naturally been found in some part of it. Cells

¹ The observations recorded in this paper were made principally at the University of Pennsylvania during the writer's tenure of a National Research Council Fellowship.

with clear polar views of metaphase chromosomes have been found in the brain, neural tube, optic cup, auditory vesicle, connective tissue, heart muscle, blood, amnion, gonads and in tissue cultures of muscles.

The Chromosome Number.—The difficulties involved in determining the exact number of chromosomes in the chick have been described in detail (5). Briefly, the trouble encountered may be said to be due, first to the extreme smallness of the shortest chromosomes of the complex making observation at times uncertain and second, to the apparent failure of the component granules of the smaller chromosomes to unite or at least to clearly indicate their proper relations to each other until late metaphase if indeed it really and always happens then.

The average number of chromosomes in the soma of the chick, based on 78 counts, is 33. I think that this is perhaps lower than the actual number which the most satisfactory counts indicate to be about 35 or 36. This variation in the chick is apparently due to a failure of the chromomeres or parts of the smaller chromosomes to unite rather than to fragmentation as in the case of the pig (1). This opinion is based upon the observations in the chick of larger numbers of distinct chromatin bodies (with occasional visible connecting threads between them) in the prophase than could be found in cells in later stages of mitosis whereas in the pig a reduction in the chromosome number as the metaphase was neared did not occur. The smaller chromosomes of the chick are the only ones concerned while in the pig the long ones are the ones that become broken up.

The Chromosome Form.—All of the longer chromosomes (about 12 in number) of the somatic cells are in the form of J's while the shorter ones are rods. Their structure and size relations (5) are alike in all the tissues studied including tissue cultures (4). The largest chromosome (chromosome pair in the male) of the complex has been shown to be the one associated with sex and is found in all somatic cells as clearly as in the cells of the gonads (5), Figs. 1 to 9. The female embryonic cells are heterozygous for this chromosome while the male cells are homozygous. No differences in the chromosomes or their behavior have been noted between any of the body tissues or in comparison with those of the embryonic germ cells.

DISCUSSION.

The morphological data on the behavior of the chromosomes in the developing embryo, although admittedly scant, has so far given no clue to the manner in which the chromosomes may contribute their potentialities to the growing organism. Before a study of somatic chromosomes had been made it seemed reasonable to expect to find the various highly differentiated cells of the body with chromosome numbers, morphology or behavior at variance both with those found in other tissues and with the specific number and general characteristics found in the gonads. This has been found not to be the case in at least three forms, the pig (1), the evening primrose (2) and the chick (5). There was some fragmentation of the somatic chromosomes in the first two forms but it was shown that none of the chromatin was lost. Furthermore this fragmentation was neither specific for any particular tissue nor constant in amount. In general the chromosome situation in the soma seems to be entirely similar to that found in the unreduced gonad cells. This is a matter difficult to understand in the present state of our information. Of all the characteristics that must be controlled or borne by or at least associated with the chromosomes (as shown by the studies on *Drosophila*) few have any chance for expression in the majority of the somatic cells and tissues and must therefore be inhibited in one way or another. The cells of the lower forms of animal life largely retain the germ-like power of reproducing the portion lost or even an entire organism following injury. This power is perhaps even more marked in plants. As differentiation becomes more extreme in the animal kingdom the ability to regenerate a part or a whole animal from cells already specialized becomes less and less until in the highest types of animals somatic cells are usually able to produce only somatic cells like themselves. Yet the chromosomes in these highly specialized cells have, in the examples studied, been found to be entirely similar to those in the germ cells containing the possibilities for a complex animal or plant. This may suggest that the chromosomes are functionless in the differentiated soma. Or it may be that having contributed their share in the production of the specialized tissue are thereafter inactive as far as are concerned the general somatic attributes or determiners

with which originally they must have been equipped. If the latter interpretation is correct the perpetuation of the complete mitotic mechanism in the soma as it exists in the reproductive organs may seem, as far as any need for an exact division of the genes is concerned, somewhat unnecessary, to be classed possibly with vestigial organs and having no more significance. In view of the great delicacy of the mechanism that exists in all cells for accurately dividing the chromatin the last suggestion does not seem impressive. But as far as our morphological data on the behavior of somatic chromosomes go, together with the behavior of the soma in growth and regeneration, the above suggestion is at least a possibility to be considered in our attempt to get at the physiology of development and genetics.

SUMMARY.

1. The chromosome number in the somatic cells of the chick is about 35 or 36.

2. No characteristic differences between the number, the sizes, the morphology or the behavior of the chromosomes in comparison either with each other or with the cells of the gonads have been noted.

3. In view of the entire similarity of the somatic and germinal mitotic behavior and in consideration of the complete inability of highly specialized cells to regenerate other than cells similar to themselves it is tentatively suggested as a basis for future discussion that the somatic chromosomes, as far at least as their genetic function is concerned, have either become functionless or their cytoplasmic environment is incapable of reacting to the possibilities presumably carried by them.

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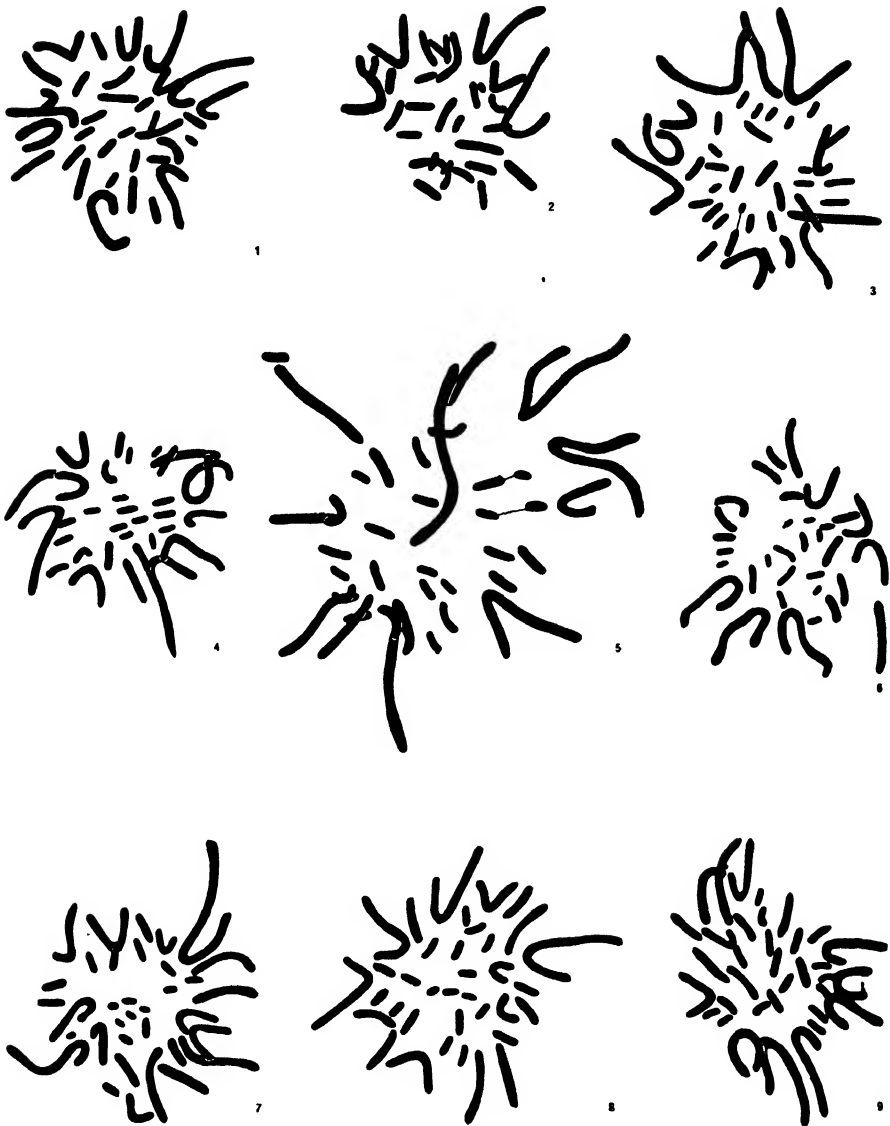
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DESCRIPTION OF PLATE 1.

All figures were drawn at table level with a Zeiss 1.5 mm. apochromat and a 15 × Orthoscopic ocular. They are reproduced at the size originally drawn. All are polar views of metaphase plates in chick embryonic cells with the exception of Fig. 5.

- FIG. 1. From optic cup. 35 chromosomes.
- FIG. 2. From amnion. 30 chromosomes.
- FIG. 3. From a male gonad. 39 chromosomes.
- FIG. 4. From neural tube. 32 chromosomes.
- FIG. 5. Late prophase in connective tissue cell grown in a tissue culture. 38 chromosomes.
- FIG. 6. From heart muscle. 33 chromosomes.
- FIG. 7. From brain. 35 chromosomes.
- FIG. 8. From female gonad. 36 chromosomes.
- FIG. 9. From neural tube. 35 chromosomes.



(Hance: Chromosomes of chick soma.)

DISSOCIATION OF POLYVALENT SUBSTANCES.

I. RELATION OF CONSTANTS TO TITRATION DATA.

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I. Notation.—The notation used in this article is as follows.

Constants.— K_1' , K_2' , K_3' , etc. = "Dissociation constants" of a polyvalent acid base or ampholyte, not corrected for activity, and representing the "steps" in formation of more negative ions from less negative ions (or molecules). These constants do not refer to groups.

G_1' , G_2' , G_3' , etc. = "Titration constants" obtained by calculating the titration data as if the solution contained monovalent acids in equivalent quantities

K_1 , K_2 , K_3 , etc. = Dissociation constants corrected for activity.

G_1 , G_2 , G_3 , etc. = Titration constants corrected for activity.

K_0^I , K_0^{II} , K_0^{III} , etc. = "Intrinsic constants" of the individual groups.

Concentrations (lower case letters).— α_1 , α_2 , α_3 , = Probabilities of dissociation according to G_1' , G_2' and G_3' , respectively.

u = Fraction of a substance in un-ionized form.

m = Fraction of a substance in mono-ionic form (mono-ion singly charged ion).

d = Fraction of a substance in di-ionic form (di-ion doubly charged ion).

i = Fraction of a substance in tri-ionic form (tri-ion triply charged ion).

n = Fraction of a substance in the more negative form (predominating at a lower P_H than P_K).

p = Fraction of a substance in the more positive form (predominating at a higher P_H than P_K).

c = Molal concentration of substance.

a = Molal concentration of strong acid.

b = Molal concentration of strong base.

b' = "Corrected equivalents of base" (see Equation 34).

b' = $(b - a + h - oh)$ in Equation 41.

\bar{b}'' = $1 - (b - a + h - oh)$ in Equation 42.

h = Hydrogen-ion concentration.

oh = Hydroxyl-ion concentration.

Activities (capital letters).— H = Hydrogen-ion activity.

OH = Hydroxyl-ion activity.

Miscellaneous.— $\left(\frac{f_0}{f_1}\right)$, $\left(\frac{f_0}{f_2}\right)$, $\left(\frac{f_0}{f_3}\right)$, etc. = Ratios of activity coefficients before

and after dissociation of one, two, three, etc. groups.

τ = Activity coefficient ratio for a single step in ionization.

z = Number between 1 and 2, in Equation 28.

v = Valence.

y = Number of groups which ionize as bases (amino groups).

Q = Special factor for Equation 38.

$Px = -\log X$ where X is any constant or variable.

E = Constant in Equation 28.

L = Function of electrostatic work between groups.

A and B = Special functions in Equations 11 to 13.

"Dissociation" refers to the effect of ionization of the acid groups and hydrolysis of the salts of basic (amino) groups.

II. Introduction.—The ionization of a divalent acid may be represented by the classical equations in the following form (see table of notations),

$$K_1' = \frac{HA^-}{H_2A} = \frac{m}{1-m-d} \quad (1)$$

$$K_2' = \frac{A^{2-}}{HA^-} = \frac{d}{m} \quad (2)$$

in which K_1' and K_2' are the classical dissociation constants. Similar equations apply to acids of higher valence.

The titration data of an acid consist of measurements of hydrogen-ion activity (H) when different concentrations of strong base (b) have been added to a concentration (c) of the acid. It is desirable to know the relationship between the titration data and the dissociation constants.

Various formulas have been derived by Larsson,¹ Harris,² Hammarsten³ and by Auerbach and Smolczyk⁴ for calculating dissociation constants from titration data. These formulas are cumbersome, limited in application, and sometimes incorrect.

On the other hand, it is well known that the titration curve of a divalent acid (see Fig. 1) *resembles* that of a mixture of two monovalent

¹ Larsson, *Z. anorg. allgem. Chem.*, **125**, 281 (1922).

² Harris, (a) *J. Chem. Soc.*, **123**, 3294 (1923); (b) *Proc. Roy. Soc.*, **95B**, 440 (1923).

³ Hammarsten, *Biochem. Z.*, **144**, 383 (1924).

⁴ Auerbach and Smolczyk, *Z. physik. Chem.*, **110**, 65 (1924).

acids. In fact, we may calculate the titration data as if there were actually two monovalent acids present,⁵ and we obtain ("titration") constants G_1' and G_2' which are the dissociation constants such acids would have.

$$G_1' = H \frac{\alpha_1}{1 - \alpha_1} \quad G_2' = H \frac{\alpha_2}{1 - \alpha_2} \quad (3)$$

These constants are not identical with the dissociation (K') constants of the divalent acid (owing to the fact that the first step of ionization may not be complete when the second begins). There has been no theoretical reason for considering these as true constants.

It is the purpose of this article to show that (under certain conditions of ionic strength) these titration (G') constants are true constants, and are related to the classical dissociation constants (K') by the equations

$$K_1' K_2' = G_1' G_2' \quad (4)$$

and

$$K_1' = G_1' + G_2' \quad (5)$$

from which it follows that

$$1/K_2' = 1/G_1' + 1/G_2' \quad (6)$$

and

$$\frac{K_1'}{K_2'} = \frac{G_1'}{G_2'} + \frac{G_2'}{G_1'} + 2 \quad (7)$$

and that similar formulas may be applied to *any* polyvalent acid base or ampholyte.

The calculation of the titration constants from titration data, is very simple (see section VIII). The above formulas then give us the dissociation constants.

III. Proof.—Suppose we have one solution containing a divalent acid with constants K_1' and K_2' and another solution containing the same concentration of each of two monovalent acids with constants

⁵ This calculation is very simple, even for a complex substance. Either the "buffer value" method of Van Slyke may be used, or the direct formulas presented in this article (section VIII).

G_1' and G_2' . If we add b' equivalents of alkali to both solutions, they will have the same P_H , providing

$$b' = m + 2d = \alpha_1 + \alpha_2 \quad (8)$$

where, according to the mass law (see Equations 3)

$$\alpha_1 = \frac{G_1}{H + G_1'}, \text{ and } \alpha_2 = \frac{G_2'}{H + G_2'} \quad (9)$$

From Equations 1, 2, 8 and 9

$$b' = \frac{HK_1' + 2K_1'K_2'}{H^2 + HK_1' + K_1'K_2'} = \frac{H(G_1' + G_2') + 2G_1'G_2'}{H^2 + H(G_1' + G_2') + G_1'G_2'} \quad (10)$$

The two solutions can have the same titration data at all values of b' only if Equation 10 holds at all values of H . Let us substitute

$$A = K_1'; B = K_1'K_2'; A' = G_1' + G_2' \text{ and } B' = G_1'G_2' \quad (11)$$

Since A must be independent of H we may solve for A and place its partial differential with respect to H equal to zero.

$$\frac{\partial A}{\partial H} = \frac{(H^2 + HA' + B')(B - B')}{(H^2 - B')^2} = 0, \text{ or } B = B' \quad (12)$$

Similarly

$$\frac{\partial B}{\partial H} = \frac{(H^2 + HA' + B')(A' - A)}{(2H + A')^2} = 0, \text{ or } A = A' \quad (13)$$

This gives us Equations 4 and 5, and proves that Equations 4 to 7 are correct.

IV. Unsymmetrical Divalent Acids.—E. Q. Adams⁶ (using different notation) stated in 1916 that for an unsymmetrical acid

$$K_1 = K_0^I + K_0^{II} \text{ and } K_2 \leq \frac{K_0^I K_0^{II}}{K_0^I + K_0^{II}} \quad (14)$$

where K_0^I and K_0^{II} are intrinsic constants of the individual groups.

Multiple ionization involves electrostatic work and work in distortion of the molecules. If L_1 and L_2 are functions of this work in the

⁶ Adams, *J. Am. Chem. Soc.*, **38**, 1503 (1916).

first and second steps, respectively, we may show⁷ that (disregarding activity correction)

$$K_1 = (K_o^I + K_o^{II}) L_1 \text{ and } K_2 = \left(\frac{K_o^I K_o^{II}}{K_o^I + K_o^{II}} \right) L_2 \quad (15)$$

Since these work functions are independent of H, K_1 and K_2 are true constants as defined by Equations 1 and 2. Therefore, Equations 3, 4 and 5 apply also to unsymmetrical acids.

In the special case of a divalent acid having very distant groups, L_1 and L_2 would each equal unity; then K_o^I would equal G_1 and K_o^{II} would equal G_2 . Otherwise there is no simple relation.

V. Polyvalent Acids.—The reasoning in Sections III and IV may be used to show that if the titration data of a trivalent acid be calculated as if there were three monovalent acids present in equivalent quantities, we will obtain three titration constants G_1' , G_2' and G_3' which are related to the dissociation constants as follows,

$$K_1' = G_1' + G_2' + G_3' = \Sigma G' \quad (16)$$

$$K_1' K_2' = G_1' G_2' + G_1' G_3' + G_2' G_3' = \Sigma G' G' \quad (17)$$

$$K_1' K_2' K_3' = G_1' G_2' G_3' = \Sigma G' G' G' \quad (18)$$

where $K_2' = \text{Equation 17 divided by 16}$
and $K_3' = \text{Equation 18 divided by 17, or}$

$$\frac{1}{K_3'} = \frac{1}{G_1'} + \frac{1}{G_2'} + \frac{1}{G_3'} = \Sigma \frac{1}{G'} \quad (19)$$

Similarly, for higher polyvalent acids.

When the constants are very different in value (for example, when $K_1' > 1000 K_2'$; when $K_2' > 1000 K_3'$; etc.) these equations become

$$K_1' = G_1'; K_2' = G_2'; K_3' = G_3'; \text{ etc.} \quad (20)$$

or, in general, "isolated" titration constants are equal to the corresponding dissociation constants.

VI. Bases and Ampholytes.—On the addition of alkali, a weak acid ionizes, while the salt of a base hydrolyzes. There is nothing in the

⁷ These equations, and also Equations 24 to 26 are simplified from some which will be derived in the second paper.

titration data to distinguish one from the other⁸ (aside from the effect of altering the ionic strength or the dielectric constant). Hence, *the above formulas for polyvalent acids apply also to bases and ampholytes*. For the ionization of a monovalent acid, or an acid group in a polyvalent substance we may write

$$K_0L = \text{H}^+ \frac{A^-}{\text{HA}} \left(\frac{f_1}{f_0} \right) \quad (21)$$

and for the hydrolysis of the salt of a monovalent base or an amino group in a polyvalent substance

$$K_0L = \text{H}^+ \frac{R \cdot \text{NH}_2}{R \cdot \text{NH}_3^+} \left(\frac{f_1}{f_0} \right) \quad (22)$$

We may define "dissociation" as referring to either process, since a hydrogen ion is removed in passing from a low *PH* value to a high *PH* value, in either case, or

$$K_0L \left(\frac{f_0}{f_1} \right) = \text{H}^+ \frac{n}{p} \quad (23)$$

where K_0 is the intrinsic constant of the group, L is a function of electrostatic work between groups (see subsequent article), (f_0/f_1) is the ratio of activity coefficients of the more *positive* ionic form p (predominating at a lower *PH* value) and of the more *negative* form n (predominating at a higher *PH* value).

The dissociation (and titration) constants of a polyvalent substance *do not* correspond to individual chemical groups (except when widely different numerically) although some one group predominates in determining the value of each constant and the number of these constants equals the number of groups. We may write for a trivalent acid, base, or ampholyte (compare with Equations 16, 17 and 18)

$$K_1' = K_0^{\text{I}} L^{\text{I}} \left(\frac{f_0}{f_1} \right)^{\text{I}} + K_0^{\text{II}} L^{\text{II}} \left(\frac{f_0}{f_1} \right)^{\text{II}} + K_0^{\text{III}} L^{\text{III}} \left(\frac{f_0}{f_1} \right)^{\text{III}} \quad (24)$$

$$K_1'K_2' = K_0^{\text{I}} K_0^{\text{II}} L^{\text{I II}} \left(\frac{f_0}{f_2} \right)^{\text{III}} + K_0^{\text{I}} K_0^{\text{III}} L^{\text{I III}} \left(\frac{f_0}{f_2} \right)^{\text{I III}} + K_0^{\text{II}} K_0^{\text{III}} L^{\text{II III}} \left(\frac{f_0}{f_2} \right)^{\text{II III}} \quad (25)$$

⁸ Bjerrum, *Z. physik. Chem.*, **104**, 147 (1923).

$$K_1' K_2' K_3' = K_0^I K_0^{II} K_0^{III} L^{I II III} \left(\frac{f_0}{f_s} \right)^{I II III} \quad (26)$$

where K_2' equals Equation 25 divided by Equation 24 and K_3' equals Equation 26 divided by 25; $\left(\frac{f_0}{f_1}\right)$, $\left(\frac{f_0}{f_2}\right)$ and $\left(\frac{f_0}{f_3}\right)$ are the activity coefficient ratios between initial and final states when one, two or three groups have "dissociated." The groups are indicated by Roman numerals. The values of L involve electrostatic work between groups.

It is advisable to designate the constants of a polyvalent substance in order of numerical value, regardless of the group which predominates in determining each value; thus, $G_1' > G_2' > G_3'$ etc. and $K_1' > K_2' > K_3'$, etc.

VII. Correction for Inter-ionic Attraction.—For a monovalent acid, or an acid group, we may write

$$\left(\frac{f_0}{f_1} \right) = \tau \quad (27a)$$

and for a monovalent base or a basic group,

$$\left(\frac{f_0}{f_1} \right) = \frac{1}{\tau} \quad (27b)$$

where τ represents the activity-coefficient ratio for a single step in *ionization* and may be estimated by the approximate equation,⁹

$$\log \tau = E \sqrt{\Sigma i \nu^z} = 0.30 \sqrt{\Sigma i \nu^z} \quad (28a)$$

where E is a constant (equal to about 0.30 at 25° and dielectric constant of 80) and $\Sigma i \nu^z$ is the sum of all the ion concentrations, each multiplied by the z power of its valence.¹⁰

⁹ Brönsted and LaMer, *J. Am. Chem. Soc.*, **46**, 555 (1924). Debye and Hückel, *Physik. Z.*, **24**, 185 (1923). Noyes, *J. Am. Chem. Soc.*, **46**, 1080, 1098 (1924).

¹⁰ When the charged groups in a polyvalent ion are very close together in space, we may multiply its concentration by the *square* of its valence (thus: $i \nu^2$) but when the groups are very far apart the concentration should be multiplied by the *first power* of its valence (thus: $i \nu$). The true value of $\log \tau$ for a solution containing a polyvalent ion, may be obtained by using for that ion, a value equal to $i \nu^z$ where z is a value between 1 and 2 and is dependent upon the concentration and the distance between the groups. There is no direct mathematical relation between the value of z and the concentration and distance, although an empirical relation might be found. In Table I, $z = 1$.

$\Sigma i \nu^2$ equals *twice* the "ionic strength" as defined by Lewis.

For the hydrogen ion, E is more nearly equal to 0.20:

$$\log \tau_{\text{H}} = \log \frac{h}{H} = 0.20 \sqrt{\Sigma i\nu^2} \quad (28b)$$

If we consider a polyvalent acid (or a polyvalent base), we may assume that the activity coefficient ratios (f_0/f_1) are all equal to each other and equal to τ (or to $1/\tau$) in Equation 24. Then, if we assume (f_0/f_2) = τ^2 (or $1/\tau^2$) and (f_0/f_3) = τ^3 (or $1/\tau^3$), we may write for a given dissociation constant and titration constant

$$K_1 = K\tau \text{ and } G' = G\tau \text{ for a polyvalent acid} \quad (29a)$$

$$K' = K/\tau \text{ and } G' = G/\tau \text{ for a polyvalent base} \quad (29b)$$

where K is the corrected constant and G is defined by these equations. If we substitute Equation 29 in Equations 24 to 26 or in 5 and 6 and 16 to 19 we find that the τ values cancel out. Hence in these equations G may be substituted for G' and K for K' , for polyvalent acids or bases—but *not for ampholytes*. Equations 4 and 18 are the only ones which apply to the corrected constants in ampholytes.

The logarithmic form of the mass-action equation for a titration constant is

$$P_{G'} = P_H - \log \frac{\alpha}{1 - \alpha} \quad (30)$$

Hence, from Equation 29:

$$P_G = P_H - \log \frac{\alpha}{1 - \alpha} \pm \log \tau = P_{G'} \pm \log \tau \quad (31)$$

where the \pm sign is positive for an acid and negative for a base. $\log \tau$ has the value given in Equation 28a. This equation may be applied to polyvalent acids and polyvalent bases, but only to those ampholytes which have constants widely different in value. An ampholyte is considered as an acid above the iso-electric point and as a base below, regardless of the intrinsic constants of the individual groups.

VIII. Calculation of Constants from Titration Data.—For electrical neutrality in a solution of a polyvalent acid, base or ampholyte

$$h + b + y c = a + o h + \alpha_1 c + \alpha_2 c + \alpha_3 c + \text{etc.} \quad (32)$$

Hence,

$$b' + y = \alpha_1 + \alpha_2 + \alpha_3 + \text{etc.} = \Sigma \alpha \quad (33)$$

where

$$b' = \frac{b - a + h - oh}{c} \quad (34)$$

is the "corrected equivalents of base." In the last equation the activities of the hydrogen and hydroxyl ions may be used in place of their concentration, with a small error.

$$b' = \frac{b - a}{c} + \frac{H - OH}{c} \text{ (approx.)} \quad (35)$$

By means of Equations 9, 33, 34, 30, 31 and 28 we may calculate the titration constants of any polyvalent acid base or ampholyte; and these may be converted into the dissociation constants by Equations 5 and 6 or 16 to 20. The complete procedure is as follows.

First.—Plot the experimental values of P_H against b' (Equation 34) and estimate graphically (with a paper mold cut the shape of a typical monovalent dissociation curve; Equation 9) the approximate P_G' values. "Monovalent curves" are so drawn between the integral values of b' that their resultant curve is identical with the experimental curve. The midpoint ($\alpha = 0.5$) of each "monovalent curve" represents a P_G' value. These tentative values may now be used to obtain, at each P_H value, approximate values of $\alpha_1, \alpha_2, \alpha_3$, etc. The latter will be used in the next three steps to obtain accurate P_G' values.

Second.—To calculate a given P_G' (e.g., $P_G^{1'}$) we must calculate, at the experimental points within its buffer range (using Equation 9), the α values ($\alpha_1, \alpha_3, \alpha_4$, etc.) corresponding to all the other (tentative) P_G' values ($P_G^{1'}, P_G^{3'}, P_G^{4'}$, etc.).

Third.—By subtracting these from $b' + y$ (Equation 33) find the α value of the P_G' in question.

Fourth.—Equation 30 gives the P_G' value.

Fifth.—To correct for activity use Equations 31 and 28a.

Sixth.—To find the dissociation constants, use Equations 5 and 6 or 16 to 20, or better still (for a divalent acid), by the following logarithmic forms of Equations 5 and 6:

$$P_{K1'} = P_{G1'} - \log \left(1 + \frac{G_2'}{G_1'} \right) \quad (36)$$

$$P_{K2'} = P_{G2'} + \log \left(1 + \frac{G_2'}{G_1'} \right) \quad (37)$$

In Fig. 2 the values of $\log [1 + (G_2'/G_1')]$ may be found for known values of $\log K_1'/K_2'$ or $\log G_1'/G_2'$. Remember that these equations apply also to the corrected constants (K and G) of acids and of bases—but not of ampholytes.

Seventh.—Correct for activity Equations 31 and 28a, if the correction was not made on the titration constant (fifth step).

In the second and fourth steps the relation between a P_G value and an α value (at a given P_H value) can be most readily found by a chart in which $P_H - P_G'$ is plotted against α (Equation 30).

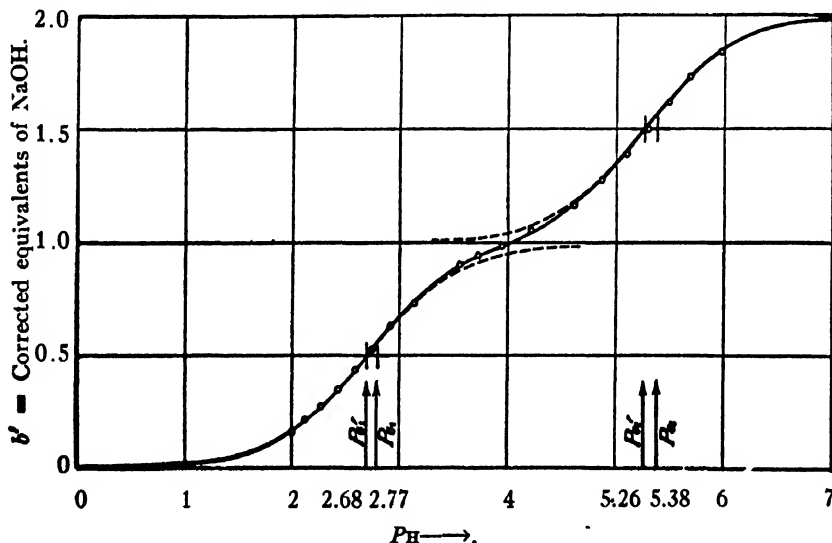


Fig. 1. Titration curve of malonic acid at 25°.

This method of calculation of titration constants is illustrated in the case of malonic acid. See Table I and Fig. 1. The concentration was constant throughout and the P_H measurements were made in a water-jacketed hydrogen electrode.¹¹ This method has been used by the author on various other substances.¹²

See Table II and Fig. 2 for relative values of dissociation constants and titration constants.

IX. Other Formulas.—The following formulas are presented for convenience.

¹¹ Simms, *J. Am. Chem. Soc.*, **45**, 2503 (1923).

¹² (a) Levene, Simms and Pfaltz, *J. Biol. Chem.*, **61**, 445 (1924). (b) Levene and Simms, *ibid.*, **63**, 351 (1925); **65**, (c) 31, (d) 519 (1925).

The hydrogen-ion activity of a solution of any divalent acid is (from Equations 9 and 32)¹³

$$\text{H} = Q \left(\sqrt{1 + G_1 \frac{c + \alpha_2 c - (b - a)}{Q^2}} - 1 \right) \quad (38)$$

$$\text{where } Q = \frac{G_1}{2} + \frac{b - a - \alpha_2 c}{2\tau_{\text{H}}} = (\text{approximately}) \frac{G_1 + b - a - \alpha_2 c}{2}$$

The value of α_2 is obtained from approximate values of H (Equation 3).

For a monovalent acid, $\alpha_2 = 0$. For a trivalent acid, substitute $(\alpha_2 + \alpha_3)$ for α_2 . For a pure solution of acid, $b - a = 0$. When $b - a > 1$, interchange Subscripts 1 and 2.

For a base or ampholyte, substitute $(yc + b - a)$ for $(b - a)$.

The concentration of base required to bring a polyvalent substance to a given P_{H} value is

$$b - a = c (\alpha_1 + \alpha_2 + \alpha_3 + \text{etc.}) - h + oh - yc \quad (39)$$

If we have an impure substance or a solution of unknown concentration it is necessary to calculate the value of c . The concentration of a polyvalent substance is

$$c = \frac{b - a + h - oh}{\alpha_1 + \alpha_2 + \alpha_3 + \text{etc.} - y} \quad (40)$$

If the G' values are unknown we cannot calculate the α values. However, for a *monobasic acid* (or a group sufficiently isolated to act like a monobasic acid), we may take values of H and of b at two points.

$$c = \frac{b_1'' b_2'' (\text{H}_1 - \text{H}_2)}{\text{H}_1 b_1'' - \text{H}_2 b_2''} \quad \text{where } b'' = (b - a + h - oh) \quad (41)$$

or if \bar{b}_1'' and \bar{b}_2'' are moles of alkali (determined volumetrically) required to complete the titration at two points,

$$c = \frac{\bar{b}_1'' \bar{b}_2'' (\text{H}_1 - \text{H}_2)}{\text{H}_1 \bar{b}_2'' - \text{H}_2 \bar{b}_1''} \quad \text{where } \bar{b}'' = 1 - (b - a + h - oh) \quad (42)$$

¹³ The value of oh in Equation 32 is neglected here.

TABLE I.

Titration of Malonic Acid at 25°.

Each solution was 0.0440 *M* with respect to malonic acid, and contained an amount of sodium hydroxide indicated in Col. 2. Standard: 0.100 *M* HCl equals *P*_H 1.090; saturated potassium chloride junction assumed constant.

1	2 Step equations	3 29b $\log \frac{[H^+]}{[H_2A]} = \frac{1}{2} \log \frac{[H^+]}{[H_2A]}$	4 29a $\log \frac{[H^+]}{[H_2A]} = \frac{1}{2} \log \frac{[H^+]}{[H_2A]}$	5 29b <i>P</i> _H	6 <i>k</i> × 10 ⁴	7 $\frac{1}{4}$	8 31 $\frac{1}{4} + \frac{1}{4} = \frac{1}{2}$	9 II 9 $\frac{(\frac{1}{2}G) + H}{\frac{1}{2}G}$	10 III 31 $\frac{(10)}{10} = 1$	11 IV 27 <i>G</i> _d	12 V 28-9 <i>G</i> _d	13 II 9 $\frac{(\frac{1}{2}G) + H}{\frac{1}{2}G}$	14 III 31 $\frac{10}{10} = 1$	15 IV 27 <i>G</i> _d	16 V 28-9 <i>G</i> _d
2.01	-0.076	0.06	0.09	1.95	1120	0.255	0.179	0.001	0.178	2.67	2.76				
2.13	+0.038	.06	.09	2.07	851	.194	.232	.001	.231	2.65	2.74				
2.28	.150	.06	.09	2.22	603	.137	.287	.001	.286	2.68	2.77				
2.44	.264	.06	.09	2.38	417	.095	.359	.001	.358	2.69	2.78				
2.59	.377	.06	.09	2.53	295	.067	.444	.002	.442	2.69	2.78				
2.75	.490	.06	.09	2.69	204	.046	.536	.003	.533	2.69	2.78				
2.92	.604	.06	.09	2.86	138	.031	.635	.004	.631	2.68	2.77				
3.14	.716	.06	.09	3.08	83	.019	.735	.009	.726	2.72	2.81				
3.56	.898	.06	.09	3.50	32	.007	.905	.018	.887	2.67	2.76				
3.73	.943	.06	.09	3.67	21	.005	.948	.026	.922	(2.66)	(2.75)				
3.95	.988	.06	.09	3.89	13	.003	.991	.042	.949	(2.67)	(2.76)				
4.21	1.056	.06	.10	4.15	7	.002	1.058					0.970	0.088	(5.22)	
4.61	1.169	.06	.10	5.55	3	.001	1.170					.987	.183	5.26	5.36
4.87	1.282	.07	.11				1.282					.993	.289	5.26	5.37
5.10	1.395		.12				1.395					.995	.400	5.28	5.40
5.30	1.508		.12				1.508					.997	.511	5.28	5.40
5.49	1.623		.12				1.623					.998	.625	5.27	5.39

The *iso-electric point* of a simple (mono-monovalent) ampholyte (if we define it as the point of equal concentrations of the ionized acidic and basic groups) is

$$H = I = \sqrt{G_1' G_2'} = \sqrt{K_1' K_2'} \quad (43)$$

For a polyvalent ampholyte having y amino groups,

$$H = I = \sqrt{K_y \cdot K_{y+1}} \text{ (approximately)} \quad (44)$$

The exact expression in an ampholyte with two acid groups and two amino groups is

$$H = I = \sqrt{K_2' K_1' + \frac{2K_2' K_3' K_4'}{H} - \frac{2H^3}{K_1'}} \quad (45)$$

where the second term on the right-hand side is negligible when K_4' is very small, and the last term is negligible when K_1' is very large. For the values of H on the right-hand side we may substitute, approximately, $\sqrt{K_2' K_3'}$.

$$H = I = \sqrt{K_2' K_3' + 2 \sqrt{K_2' K_3'} \left(K_4' - \frac{K_2' K_3'}{K_1'} \right)} \quad (46)$$

The expressions involving the titration constants are more complicated.¹⁴

The "*Buffer Value*" method of Van Slyke, for determining titration constants is advantageous for certain complex substances. By using Equations 32 and 9, and differentiating, we get for the "buffer value" of any substance at any P_H value:

$$\beta = \frac{db}{dP_H} = 2.3_{HC} \left[\frac{G_1'}{(H + G_1')^2} + \frac{G_2'}{(H + G_2')^2} + \text{etc.} \right] + 2.3 (h + oh) \quad (47)$$

when $P_H = P_{G_1'}$, we get

$$\frac{2.3_{HC} G_1'}{(H + G_1')^2} = \frac{2.3 c}{4} = 0.575 c \quad (48)$$

¹⁴ See Levene and Simms [*J. Biol. Chem.*, **55**, 801 (1923)], Equation 15a. Since the notation in that article is different, we must substitute: $G_1' = k_{b1}$; $G_2' = k_{b2}$; $G_3' = K_{a1}$; and $G_4' = K_{a2}$.

This method is described by Van Slyke¹⁵ and by Hastings and Van Slyke.¹⁶ β is the slope of the curve when b is plotted against P_H .

As a modification, we may define the "corrected equivalent buffer value" as the slope of the curve when b' (instead of b) is plotted against P_H :

$$\frac{db'}{dP_H} = \Sigma\beta' = \beta_1' + \beta_2' + \beta_3' + \text{etc.} = (\text{approximately}) \frac{b_2' - b_1'}{P_{H_2} - P_{H_1}} \quad (49)$$

where

$$\beta_1' = \frac{2.3 \text{ } H G_1'}{(H + G_1')^2} = 2.3 \alpha_1 (1 - \alpha_1)$$

$$\beta_2' = \frac{2.3 \text{ } H G_2'}{(H + G_2')^2} = 2.3 \alpha_2 (1 - \alpha_2) \quad (50)$$

and b_1' , P_{H_1} , b_2' and P_{H_2} are values at two points near each other.

TABLE II.

Values of Dissociation Constants Compared with Titration Constants.

1	2	3	4	5	6	7	8
Acid	P_{K_1}	P_{K_2}	$\log K_1/K_2$	$\log \left(\frac{G}{1+G} \right)$	P_{G_1}	P_{G_2}	$\log G_1/G_2$
Carbonic.....	6.36	10.22	3.86	0	6.36	10.22	3.86
Oxalic.....	1.42	4.35	2.93	0	1.42	4.45	2.93
Malonic.....	2.77	5.38	2.59	0	2.77	5.38	2.59
Succinic.....	4.20	5.62	1.42	0.02	4.22	5.60	1.38
Glutaric.....	4.32	5.50	1.18	.03	4.35	5.47	1.12
Adipic.....	4.43	5.62	1.19	.03	4.46	5.59	1.13
Pimelic.....	4.49	5.59	1.10	.04	4.53	5.55	1.03
Suberic.....	4.52	5.55	1.03	.05	4.57	5.50	0.93
Azelaic.....	4.60	5.56	0.96	.05	4.65	5.51	.86
Sebacic.....	4.62	5.60	.98	.05	4.67	5.55	.88

These equations may be used in a manner similar to that described for 33 and 30. The easiest way to find a G' value, when the cor-

¹⁵ Van Slyke, *J. Biol. Chem.*, **52**, 525 (1922).

¹⁶ Hastings, A. B., and Van Slyke, *ibid.*, **53**, 269 (1922).

responding β' value has been calculated, is from a chart in which β' is plotted against $P_H - P_{G'}$ (from arbitrary values of α , Equation (30) gives $P_H - P_{G'}$, and (50) gives β'). Equations 28 and 31 may be used to correct for activity of acids or bases, but not of ampholytes. The buffer value method requires more data and greater accuracy than the direct method (28 to 35) of calculating titration constants.

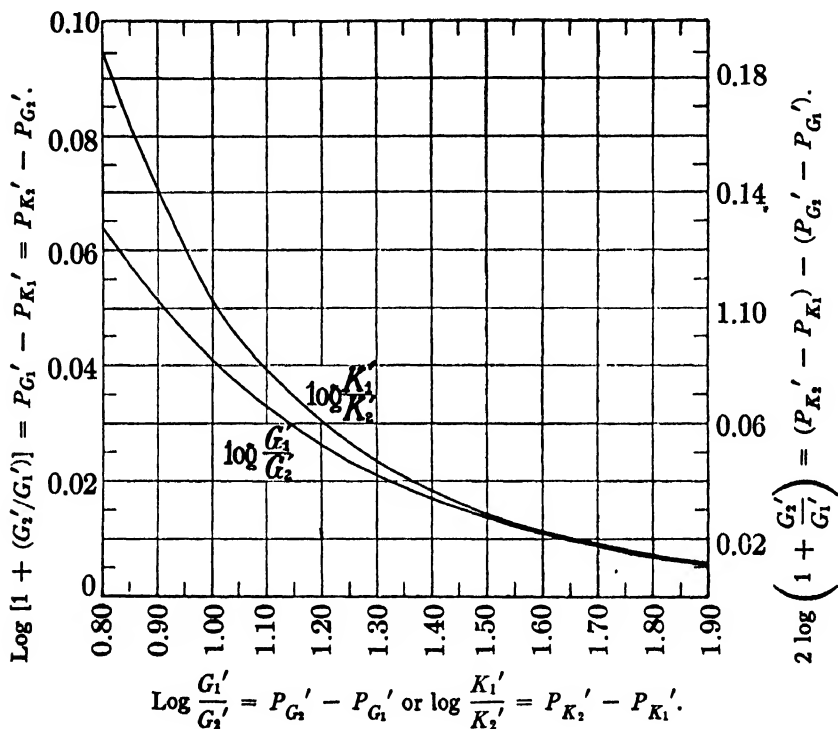


Fig. 2. Plot for finding $\log \left(1 + \frac{G_2'}{G_1'} \right)$.

X. SUMMARY.

The electrometric titration data of *any* polyvalent acid, base or ampholyte (whether "symmetrical" or not) may be calculated (by Equations 28 to 35 or 47 to 50) as if it were an equivalent mixture of monovalent acids. (See Table I and Fig. 1 for malonic acid.)

The "titration" constants (G'), thus obtained, are nearly (but not exactly) equal to the classical "dissociation" constants (K') to which they are related by simple formulas 4 to 7, 16 to 20 and 36 and 37.

(See Table II and Fig. 2.) The corresponding (G and K) constants (corrected for activity of the ions) are related to each other by the same formulas—for polyvalent acids and bases—but not for ampholytes (except in very dilute ion concentration).

This gives a method of calculating dissociation constants which is easy, accurate and general in application.

Formulas are also given for calculating the concentration of base (39) or of substance (40–42); the hydrogen-ion activity (38); and iso-electric points (43–46).

DISSOCIATION OF POLYVALENT SUBSTANCES.

II. RELATION OF CONSTANTS TO CHEMICAL STRUCTURE.

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I. Notation.—The notation used in this article is the same as in the first paper,¹ with the following additions.

Distances in Ångström units (10^{-8} cm).

"*r*" = "Distance" calculated by Bjerrum's Formula 3.

$\sum \frac{1}{r}$ = Sum of the reciprocal distances in Equation 25.

r_u = Distance between the negative charges in an *undissociated* divalent acid.

r_m = Distance between the negative charges in a *mono-ion* of a divalent acid.

r_m' = Distance between the negative charge of the ionized group and the positive charge of the un-ionized group in the *mono-ion*.

r_d = Distance between the two negative charges in a *di-ion* of a divalent acid.
(See Fig. 1.)

r_L = Distance between two *like* charges in any substance.

r_o = Distance between two *opposite* charges in any substance.

Work terms (in ergs per mole).

$W_1 = RTP_{K_1}$ = Work in first step of ionization.

$W_2 = RTP_{K_2}$ = Work in second step of ionization.

$W_0 = RTP_{K_0}$ = "Intrinsic" work in ionizing a given group in a given molecule.
This work depends upon chemical constitution and does not involve electrostatic work between ionizable groups.

ϕ = Electrostatic work in removing a hydrogen ion from the attraction of a negatively charged (ionized) group.

θ = Electrostatic work performed by two groups of like charge in repelling each other to a greater distance.

Δ = "Distortional" (electrostatic) work done in altering the relative positions of the positive and negative charges in an un-ionized group, with respect to an ionized group.

Miscellaneous.

Y = "Substitution" coefficient, of a substituted group.

¹ Simms, *J. Am. Chem. Soc.*, **48**, 1239 (1926).

x = Position of substitution.

$F\Delta$ = "Index" of distortional work. (See Equations 10 and 12.)

F = A constant = $\frac{0.4343 \cdot 10^8}{RT}$ = 0.00175 mole per erg.

J = A constant = $F \frac{e^2 N}{D}$ = 3.0 Ångström units (at 25° C., $D = 80$).

e = Charge on one electron = 4.774×10^{-10} e.s.u.

N = Avogadro number = 6.06×10^{23} molecules per mole.

D = Dielectric constant. (For water $D = 80$.)

R = Gas constant = 8.35×10^7 ergs per mole per degree.

T = Absolute temperature = 298° abs.

L = Function of electrostatic work between groups (see Equation 25).

n = Number of groups with $K_0 L$ values too large to be considered in Equations 22 to 24 and 26 to 28.

A and B = Subscripts referring to initial and final states in Equation 25.

f_0, f_1, f_2 , etc. = Activity coefficients when 0, 1, 2, etc. groups, have "dissociated."

Px = $-\log X$, where X is any constant or quantity.

Chemical groups are indicated by Roman numerals.

"Dissociation" refers to the effect of ionization of the acid groups and hydrolysis of salts of the basic (amino) groups.

S , after the number of an equation, indicates that the equation applies to symmetrical acids only.

II. Introduction.—Every polyvalent acid, base or ampholyte has a number of dissociation constants equal to the number of ionizable groups. The numerical value of each constant in water solution depends, first, upon the *nature of the groups*; second, upon the *influence of other substitutes* on each group; and third, the *effect of electrostatic forces* between the ionizable groups.

The *first factor* (the nature of the groups) determines the general order of magnitude of each constant while the other two factors have an influence to an extent dependent upon conditions. Table I gives approximate *characteristic* constants (P_{K_c}) of a few groups when attached to an aliphatic hydrocarbon or to a benzene ring.

Other groups are $-\text{SH}$, $-\text{SO}_3\text{H}$, $-\text{CN}$, etc. The constants for the amines are expressed in terms of hydrogen-ion activity (hydrolysis constants).

Concerning the *second factor*.—Each substituent produces an effect upon an ionizable group, dependent upon the nature of the substituent

and the position of its substitution. The effect of various substituents upon a —COOH group has been studied by Derrick, Flürscheim, Micheal, Walker and Wegscheider.² They concluded that the effect was *through the molecule* and was reduced to about *one-third* for each carbon atom through which it was propagated.

TABLE I.
Characteristic Constants.

Group	Aliphatic	P_{K_c}	Aromatic	P_{K_c}
—NH_2	CH_2NH_2	10.6	$\text{C}_6\text{H}_5\cdot\text{NH}_2$	4.6
=NH	$(\text{CH}_2)_2\text{NH}$	10.7		
≡N	$(\text{CH}_2)_3\text{N}$	9.7		
—COOH	$\text{CH}_2\cdot\text{COOH}$	4.73	$\text{C}_6\text{H}_5\cdot\text{COOH}$	4.2
—OH	CH_2OH	> 14	$\text{C}_6\text{H}_5\cdot\text{OH}$	9
$\text{—PO}_4\text{H}_2$	$\text{CH}_2\cdot\text{PO}_4\text{H}_2$	(2) and (7)		
$\text{=PO}_4\text{H}$	$(\text{CH}_2)_2\cdot\text{PO}_4\text{H}$	(2)		

We may express this as follows (2.7 fits the data better than 3):

$$P_{K_0} = P_{K_c} + Y/(2.7)^x \quad (1)$$

where Y is a coefficient, constant for each substituent and x is the number of atoms separating the group from the carboxyl.

The effect seems to be additive; hence, for a number of substituents with Y values, Y_1 , Y_2 , Y_3 , etc., and substituted in positions x_1 , x_2 , x_3 , etc., the "intrinsic" constant (K_0) of the group would be

$$P_{K_0} = P_{K_c} + \frac{Y_1}{(2.7)^{x_1}} + \frac{Y_2}{(2.7)^{x_2}} + \frac{Y_3}{(2.7)^{x_3}} + \text{etc.} = P_{K_c} + \sum \frac{Y}{(2.7)^x} \quad (2)$$

Approximate values of Y for various groups are given in Table II.

TABLE II.
Substitution Coefficients.

Substituent	—Cl	—Br	—I	—OH	=O	—NO_2
Y	5.4	5.2	4.5	2.5	8.0	8.3
Substituent	—COOH	—COOCH_3	$\text{—COOC}_2\text{H}_5$	—CH_3	$\text{—C}_2\text{H}_5$	—NH_2
Y	2.7	(4.2)	(3.9)	(0)	(0)	(4)

² See review by Lowry, *Trans. Faraday Soc.*, **19**, 497 (1923), and Lehfeldt, "Electro-chemistry," Longmans, Green and Co., 1918, Part I, p. 111 and following pages.

If, as has been supposed, this effect is through the molecule, it would appear that a substituent performs a certain amount of work upon the valence electrons of the adjacent carbon atom and that this work is propagated to the other three valences and distributed approximately among them. It is not within the province of this article, however, to refute or propose any theories on the mechanism of this effect. We present Equations 1 and 2 to express observations of the above-named men. Although entirely empirical, these equations have practical utility in calculating "intrinsic" constants from chemical structure.

For a monovalent acid, the dissociation constant is identical with the intrinsic constant. However, for polyvalent acids the dissociation constants (of the steps of ionization) differ from the intrinsic constants (of the chemical groups) by an amount dependent upon the electrostatic work between the groups. This, the *third factor*, will now be discussed.

III. Influence of Electrostatic Forces between Groups.—A. Formula of Bjerrum for symmetrical divalent acids.

Adams (in 1916) showed that for a long symmetrical divalent acid, the ratio K_1/K_2 should equal 4, whereas actual acids have a ratio greater than 4, the deviation being due to electrostatic forces (which were not formulated).

Bjerrum³ (in 1923) derived the following formula on the assumption that the deviation of $K_1/4K_2$ from unity is a measure of the work in removing the second hydrogen ion from the electrostatic attraction of the charge on the first group, in a mono-ion (or singly charged ion).

$$r = \log (K_1/4K_2) - P_{K_2} - P_{K_1} - 0.60 \quad (3aS)$$

or

$$P_{K_2} - P_{K_1} - 0.60 = \frac{3.1}{r} \quad (3bS)$$

The distance " r " is a vaguely defined distance expressed in Ångström units.

³ Bjerrum, *Z. physik. Chem.*, 106, 219 (1923).

The distances calculated by this expression have been shown by Bjerrum and Larson to correspond (very roughly) with those computed from the chemical structure of a number of dibasic acids.

However, this formula does not involve the distortional work Δ between the charged group and the un-ionized group in the mono-ion and, hence, gives quite inaccurate values for some acids.

It is desirable to determine the magnitude of this distortional work and also to know the relation between the constants and the dimensions in higher polyvalent acids, in bases and in ampholytes.

B. More Accurate Formulas, for all divalent acids.

The *total* work, or free energy change, in the complete ionization of a divalent acid, must equal the algebraic sum of all the work terms in passing from the initial state (low P_H) to the final state (high P_H). Hence (see Notations)

$$W_1 + W_2 = W_0^I + W_0^{II} + \phi - \theta \quad (4)$$

If Δ is the same for either group, the work in the first step of ionization is

$$W_1 = -\Delta - RT \ln (K_0^I + K_0^{II}) = -\Delta + W_0 - RT \ln P \quad (5)$$

where $P = (1 + K_0^{II}/K_0^I)$ is a probability factor (equal to 2 in a symmetrical acid).

$$\text{Hence} \quad W_2 = \Delta + \phi - \theta + W_0^{II} + RT \ln P \quad (6)$$

$$\text{and} \quad W_2 - W_1 = 2\Delta + \phi - \theta + W_0^{II} - W_0^I + 2 RT \ln P$$

$$= 2\Delta + \phi - \theta - RT \ln \frac{K_0^I K_0^{II}}{(K_0^I + K_0^{II})^2} \quad (7)$$

From the definitions of the distances r_m and r_d , the work ϕ and θ (of removing the second hydrogen ion from the charge of the other group, and of extending the molecule to the distance r_d) for a divalent acid should be

$$\phi = \frac{e^2 N}{D} \frac{1}{r_m} \text{ and } \theta = \frac{e^2 N}{D} \left(\frac{1}{r_m} - \frac{1}{r_d} \right) \quad (8)$$

$$\text{Hence} \quad \phi - \theta = \frac{e^2 N}{D \cdot r_d} = \frac{J}{F \cdot r_d} \quad (9)$$

Similarly, the distortional work Δ resulting from the effect of the charged group upon the un-ionized group in the mono-ion is equal to the work of attracting the positive hydrogen atom from its original distance r_u to its final distance r_m' , less the work of moving the negative oxygen atom from r_u to r_m :

$$F \Delta = J \left(\frac{1}{r_m'} - \frac{1}{r_u} \right) - J \left(\frac{1}{r_m} - \frac{1}{r_u} \right) = J \left(\frac{1}{r_m'} - \frac{1}{r_m} \right) \quad (10a)$$

If we assume $r_m - r_m' = 0.30$, the radius of the hydrogen ion, we get

$$F \Delta = 3.0 \left(\frac{1}{r_m - 0.30} - \frac{1}{r_m} \right) \text{ (approximately)} \quad (10b)$$

Hence, from Equation 7

$$\begin{aligned} P_{K_2} - P_{K_1} - 0.60 &= \frac{J}{r_d} + 2J \left(\frac{1}{r_m'} - \frac{1}{r_m} \right) + \log \frac{(K_o^I + K_o^{II})^2}{K_o^I K_o^{II}} \\ &= \frac{J}{r_d} + 2F\Delta + \log \frac{(K_o^I + K_o^{II})^2}{K_o^I K_o^{II}} \end{aligned} \quad (11)$$

Solving for the index of distortional work, gives:

$$F \Delta = \frac{1}{2} \left(P_{K_2} - P_{K_1} - 0.60 - \frac{3.0}{r_d} - \log \frac{(K_o^I + K_o^{II})^2}{K_o^I K_o^{II}} \right) \quad (12)$$

For a symmetrical acid these become:

$$P_{K_2} - P_{K_1} - 0.60 = \frac{3.0}{r_d} + 2F\Delta \quad (11S)$$

and

$$F \Delta = \frac{1}{2} \left(P_{K_2} - P_{K_1} - 0.60 - \frac{3.0}{r_d} \right) \quad (12S)$$

Compare Equations 11 with Bjerrum's Formula 3bS.

Equations 11 and 12 are used in conjunction with Equations 10.

Equations 4, 5 and 6, respectively, give the following equations for unsymmetrical divalent acids.

$$P_{K_1} + P_{K_2} = P_{G_1} + P_{G_2} = P_{K_0}^I + P_{K_0}^{II} + \frac{3.0}{r_d} \quad (13)$$

$$P_{K_1} = P_{K_0} - \log \left(1 + \frac{K_0^{II}}{K_0^I} \right) - F \Delta \quad (14)$$

$$P_{K_2} = P_{K_0}^{II} + \log \left(1 + \frac{K_0^{II}}{K_0^I} \right) + F \Delta + \frac{3.0}{r_d} \quad (15)$$

For symmetrical acids these become:

$$P_{K_1} + P_{K_2} = P_{G_1} + P_{G_2} = 2P_{K_0} + \frac{3.0}{r_d} \quad (13S)$$

$$P_{K_1} = P_{K_0} - 0.30 - F \Delta \quad (14S)$$

$$P_{K_2} = P_{K_0} + 0.30 + F \Delta + \frac{3.0}{r_d} \quad (15S)$$

Equations 10 to 15 may be used to calculate distances when the constants are known, or constants when the distances are known. P_{K_0} values may be calculated from Equations 13 to 15.

IV. Application of the Above Formulas to Divalent Acids.—It has been often stated that, for a divalent acid, K_1 is greater than $4K_2$ because the negative charge on the first group hinders the removal of the second hydrogen ion. If this were the only work occurring between the two groups, then (in a symmetrical acid) K_1 would equal $2K_0$.⁴ However, the data of *cis* and *trans* acids show that this work may amount to only 20–40% of the work between the groups in such acids as maleic, oxalic and malonic. The remaining 60–80% is “distortional” work in changing the relative positions of the electrostatic charges in mono-ion and in the di-ion. K_1 does not equal $2K_0$.

The above formulas include all these work terms. They involve the distances r_m and r_m' between the *like* and *opposite* charges, respectively, in the mono-ion; and the distance r_d between the *like* charges in the di-ion (see Fig. 1).

⁴ This is the basis of Wegscheider's rule that $K_1 = 2Ke$, where Ke is the constant of the half ester. This rule can be valid only when the acid is symmetrical, when $K_2 = K_0$ and when $F\Delta = 0$ (or when the errors compensate each other).

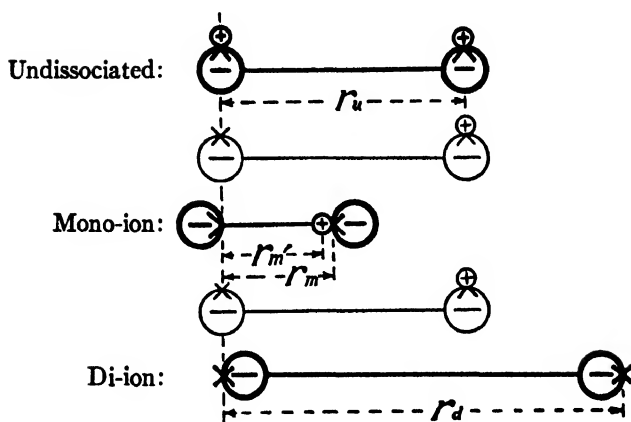


Fig. 1. Distances in a dicarboxylic acid. Only the ionizable hydrogen atoms (small circles) and the adjacent oxygen atoms (large circles) are shown. Crosses (x) indicate the position of the negative charge on the oxygen atoms. The carbon atoms (omitted) lie in an extended zigzag chain in the di-ion; and in irregular chains in the mono-ion and the undissociated molecule. The distances are approximately those of suberic acid. In Formula 25, $r_L = r_m$ and $r_0 = r_m'$ in the mono-ion; and $r_L = r_d$ in the di-ion.

TABLE III.
Calculation of r_m (and of P_{K0}) in α,ω -Dicarboxylic Acids.

1	2	3	4	5	6	7	8	9	10
Equation	Found			(3)	Estimated		(12S)	(10b)	(15S)
Acid	P_{K_1}	P_{K_2}	$P_{K_2} - P_{K_1}$	"r"	r_d	$\frac{3.0}{r_d}$	$F \Delta$	r_m	P_{K_0}
Carbonic.....	6.36	10.22	3.86	0.9	3	1.0	1.1	1.1	7.7
Oxalic.....	1.42	4.35	2.93	1.3	4.5	0.67	0.83	1.2	2.55
Malonic.....	2.77	5.38	2.61	1.5	5.7	.53	.74	1.3	3.81
Succinic.....	4.20	5.62	1.42	3.7	7.0	.43	.20	2.3	4.70
Glutaric.....	4.32	5.50	1.18	5.2	8.2	.37	.10	3	4.72
Adipic.....	4.43	5.62	1.19	5.1	9.4	.32	.13	3	4.86
Pimelic.....	4.49	5.59	1.10	6.0	10.6	.28	.11	3	4.9
Suberic.....	4.52	5.55	1.03	7.0	11.8	.25	.09	3	4.9
Azelaic.....	4.60	5.56	0.96	8.3	12.1	.25	.05	4	4.9
Sebacic.....	4.62	5.60	.98	7.9	14.3	.21	.08	4	5.0

Distances "r," r_d and r_m are in Ångström Units.

The distance r_u , in the undissociated molecule, is not included. It has no bearing upon the dissociation constants.

In Table III are to be found the P_K values of the α , ω -dicarboxylic acids. In Col. 5 are the values of " r " according to Bjerrum's Formula 3. They increase qualitatively with the length of chain—but have no quantitative significance.

In Col. 6 are values of r_d estimated from the chemical structure (see Section IV on molecular dimensions).

From these values and from the difference between the constants ($P_{K_2} - P_{K_1}$), the index of distortional work ($F\Delta$) may be calculated (from 12S). From Equation 10b we then get the values of r_m in Col. 9 (under the assumption that $r_m' = r_m - 0.30$). These values are all reasonable and possible.

Due to thermal agitation, however, the di-ion may not extend to its greatest possible length. Then the values of r_d would be less than those given; $F\Delta$ would be smaller; and the distances r_m would be slightly greater. The high values of r_m in the longer acids indicates that, although the groups can touch each other, thermal agitation prevents them from remaining in this position.

Values of P_{K_0} (Equation 13S) are given in the last column. These agree approximately with values (not given) calculated from Equation 2.

Let us now consider maleic acid (*cis*) and fumaric acid (*trans*) together with a group of isomeric *cis* and *trans* sugar acids (studied by Levene and Simms).⁵ The "distances" calculated by Bjerrum's Formula 3 are given in Col. 5, Table IV. From structural models, based upon the chemical structure, we have estimated the values of r_d and r_m (Cols. 6 and 7) in the di-ions and mono-ions. From these distances, the values of $P_{K_2} - P_{K_1}$ in Col. 10 have been calculated by Equations 10b and 11S. These correspond very well with the experimental values in Col. 11.

Still better proof of the validity of Equations 10 to 15 is found in the last column, in which isomeric *cis* and *trans* acids are found to have nearly the same intrinsic constants.

V. Molecular Dimensions.—In estimating the distances between

⁵ Levene and Simms, *J. Biol. Chem.*, **63**, 351 (1925).

parts of a molecule we may use the following values from Bragg⁶ as giving the diameters of the respective atoms (in Ångström units): C = 1.54; H = 0.60; O = 1.30.

We may assume that each atom is spherical; that the angle between any two valences in any element is about 109.5°; that adjacent atoms are free to rotate about the axis of a single bond (without work) and that a double bond is rigid.

Although these assumptions are not entirely justifiable, they give us a means for estimating the distances.

The distance r_d between ionized groups of like charge is the maximum distance compatible with the flexibility of the molecule and

TABLE IV.
Calculation of $P_{K_2} - P_{K_1}$ in Cis and Trans Acids.

1	2	3	4	5	6	7	8	9	10	11	12
	Equation	Found		(3)	Estimated			(10b)	(11S)	Found	(14S)
	Acid	P_{K_1}	P_{K_2}	"r"	r_d	r_m	$\frac{3.0}{r_d}$	$F \Delta$	$(P_{K_2} - P_{K_1})$		P_{K_0}
Cis	Maleic	1.92	6.30	0.8	6.0	0.9	0.50	1.67	4.44	4.38	3.89
Trans	Fumaric	3	4.6	3.0	6.5	3.5	.46	0.18	1.4	1.6	3.5
Cis	2,5-Anhydrosaccharic	1.98	4.94	1.3	7.0	1.2	.43	.83	2.69	2.96	3.11
	2,5-Anhydromucic	2.02	4.53	1.6	7.0	1.2	.43	.83	2.69	2.51	3.15
Trans	2,5-Anhydromanno- saccharic	2.81	3.80	7.9	7.5	4.0	.40	.06	1.12	0.99	3.17
	2,5-Anhydroidosaccharic	3.03	4.00	8.3	7.5	4.0	.40	.06	1.12	.97	3.39

Distances "r" r_d and r_m are in Ångström Units.

thermal agitation; while r_m is the minimum distance between the like charges in an ionized and an un-ionized group (see Fig. 1). We have taken r_m' equal to $r_m - 0.30$, where 0.30 is the "radius" of the hydrogen ion. (This is somewhat arbitrary, since the "radius" does not necessarily have a constant value, and the difference between r_m and r_m' may not equal the radius.)

For a simple substance these distances may be estimated graphically. When there is a ring in the molecule, or when more than two groups are ionized, the various distances cannot be easily estimated except

⁶ Bragg, *Phil. Mag.*, 40, 169 (1920).

by use of a properly made structural model.⁷ Such a model makes it possible to find the most probable shape of the molecule having certain positively charged or negatively charged groups, or both. The distances may then be measured.

VI. Trivalent Acids.—If $(\text{HA}^-)'$, $(\text{HA}^-)''$ and $(\text{HA}^-)'''$ represent the three types of di-ions produced by ionization of different pairs of groups in a trivalent acid, we may write for the first two steps in ionization:

$$K_1 K_2 = H^2 \frac{(\text{HA}^-)' + (\text{HA}^-)'' + (\text{HA}^-)'''}{H_3A} \quad (16)$$

Let $(\text{HA}^-)'$ be the concentration of the type of ion produced by ionizing Groups I and II (and not III); then the work will equal (see Equations 4 and 5)

$$W' = W_0^I + W_0^{II} + (\phi - \theta)^{I II} - \Delta^{I III} - \Delta^{II III} \quad (17)$$

where the Roman numerals indicate the groups involved in each work term.

Hence,

$$K_0^I K_0^{II} \cdot e^{\frac{\Delta^{I III} + \Delta^{II III} - (\phi - \theta)^{I II}}{RT}} = H^2 \frac{(\text{HA}^-)'}{H_3A} \quad (18)$$

Similarly for $(\text{HA}^-)''$ and $(\text{HA}^-)'''$; and we get Equation 20 (the second below). In a similar manner we derive (19) and (21):

$$K_1 = K_0^I e^{\frac{\Delta^{I II} + \Delta^{I III}}{RT}} + K_0^{II} e^{\frac{\Delta^{I II} + \Delta^{II III}}{RT}} + K_0^{III} e^{\frac{\Delta^{I III} + \Delta^{II III}}{RT}} \quad (19)$$

$$K_1 K_2 = K_0^I K_0^{II} e^{\frac{\Delta^{I III} + \Delta^{II III} - (\phi - \theta)^{I II}}{RT}} + K_0^I K_0^{III} e^{\frac{\Delta^{I II} + \Delta^{II III} - (\phi - \theta)^{I III}}{RT}} + K_0^{II} K_0^{III} e^{\frac{\Delta^{I II} + \Delta^{I III} - (\phi - \theta)^{II III}}{RT}} \quad (20)$$

⁷ A structural model set is in use in this Laboratory, consisting of spherical mahogany balls (painted different colors for different elements) proportional to the above diameters, where 4 cm. represents 1 Ångström unit (10^{-8} cm). They are held together by means of split brass pins (with a heavy coil spring at the center for ring compounds) which fit into brass lined holes in the balls. The holes are at an angle of 109.5° from each other for all elements. The number of holes equals the valence. Adjacent balls touch each other. Double bonded atoms are constructed specially.

$$K_1 K_2 K_3 = K_0^I K_0^{II} K_0^{III} e^{\frac{-(\phi - \theta)^I II - (\phi - \theta)^I III - (\phi - \theta)^II III}{RT}} \quad (21)$$

K_2 equals Equation 20 divided by Equation 19 and K_3 equals Equation 21 divided by 20.

VII. Polyvalent Acids, Bases and Ampholytes.—In order to estimate the (uncorrected) dissociation constants⁸ (K') for *any* polyvalent substance, we may write (as above)

$$K'_{n+1} = K_0^I L^I \left(\frac{f_0}{f_1}\right)^I + K_0^{II} L^{II} \left(\frac{f_0}{f_1}\right)^{II} + \text{etc.} = \Sigma K_0 L \left(\frac{f_0}{f_1}\right) \quad (22)$$

$$\begin{aligned} K'_{n+1} K'_{n+2} &= K_0^I K_0^{II} L^I L^{II} \left(\frac{f_0}{f_2}\right)^{I II} + K_0^I K_0^{III} L^I L^{III} \left(\frac{f_0}{f_2}\right)^{I III} + \text{etc.} \\ &= \Sigma K_0 K_0 L \left(\frac{f_0}{f_2}\right) \end{aligned} \quad (23)$$

$$\begin{aligned} K'_{n+1} K'_{n+2} K'_{n+3} &= K_0^I K_0^{II} K_0^{III} L^I L^{II} L^{III} \left(\frac{f_0}{f_3}\right)^{I II III} + \\ &K_0^I K_0^{II} K_0^{IV} L^I L^{II} L^{IV} \left(\frac{f_0}{f_3}\right)^{I II IV} + \text{etc.} = \Sigma K_0 K_0 K_0 L \left(\frac{f_0}{f_3}\right) \end{aligned} \quad (24)$$

and so forth. Then, K'_{n+2} = Equation 23 divided by Equation 22,⁹ K'_{n+3} = Equation 24 divided by Equation 23, etc.

The subscript n refers to the number (known or unknown) of groups having values of $K_0 L$ too large to be considered. Only those groups are considered which have values of $K_0 L$ less than $1000 K_x$ and greater than $0.001 K_x$, where K_x is the constant in question. For an acid in which all groups are considered, $n = 0$. The number of terms in each equation is equal to the number of possible combinations of the requisite number of K_0 constants.

⁸ Up to this point in this article, we have discussed the K constants (corrected for activity) and the equations are applicable to very dilute solutions. In the Equations 22 to 24, involving the (uncorrected) K' constants, we have introduced the activity ratios $\left(\frac{f_0}{f_1}\right)$, $\left(\frac{f_0}{f_2}\right)$ and $\left(\frac{f_0}{f_3}\right)$ between the activities in the initial and final states, when one, two or three groups (designated by Roman numerals) have "dissociated." These may be neglected if the K constants are substituted for K' constants of polyvalent acids or bases—but not for ampholytes (except in very dilute solution; see previous article).

The value of each L is such that

$$\log L = 3.0 \left[\left(\sum \frac{1}{r_0} - \sum \frac{1}{r_L} \right)_B - \left(\sum \frac{1}{r_0} - \sum \frac{1}{r_L} \right)_A \right] \quad (25)$$

where A is the initial state (at a low P_H , where none of the acid groups under consideration have ionized and the basic groups have not hydrolyzed); and B is the final state (at a high P_H , where the indicated groups have ionized or hydrolyzed). $\sum(1/r_L)$ is the sum of all the reciprocal distances between *like charges*, while $\sum(1/r_0)$ is the sum of all reciprocal distances between *opposite charges*. This refers to *all* charges whether in ionized or non-ionized groups.

These distances must be estimated from the structural formula of the substance (as described in Section V).

When the initial state (A) is the neutral molecule (as in the case of a polyvalent acid, when $n = 0$) we may write

$$\log L = 3.0 \left(\sum \frac{1}{r_0} - \sum \frac{1}{r_L} \right)_B \quad (25a)$$

VIII. Summary of Constants.—The dissociation, the intrinsic and the titration constants, respectively, of *any* acid base or ampholyte are related as follows.

$$K'_{n+1} = \Sigma K_0 L \frac{f_0}{f_1} = \Sigma G' \quad (26)$$

$$K'_{n+1} K'_{n+2} = \Sigma K_0 K_0 L \frac{f_0}{f_2} = \Sigma G' G' \quad (27)$$

$$K'_{n+1} K'_{n+2} K'_{n+3} = \Sigma K_0 K_0 K_0 L \frac{f_0}{f_3} = \Sigma G' G' G' \quad (28)$$

etc., where the intrinsic constant is related to the characteristic constant as follows:

$$P_{K_0} = P_{K_0} + \Sigma \frac{Y}{(2.7)^2} \quad (2)$$

and the activity correction for acids (+), and bases (−) but not ampholytes, is approximately

$$P_K = P_{K'} \pm 0.30 \sqrt{\Sigma i \nu^2} \quad (29)$$

and

$$P_G = P_{G'} \pm 0.30 \sqrt{\Sigma i \nu^2} \quad (I, 31)$$

SUMMARY.

The dissociation constants of a polyvalent acid, base or ampholyte depend, first, upon the types of ionizable groups (Table I); second, upon the influence of all substituents (Equation 2 and Table II); and finally, upon the electrostatic forces between the ionizing groups.

These forces have been formulated (Equations 10–15) in terms of the distances between the like and unlike charges in the mono-ion (singly charged ion) and also in the di-ion (doubly charged ion) of a *divalent* acid. "Distortional" work plays a large rôle in some acids. By use of these formulas the distances (r_m) between the negative charges in the mono-ions of some divalent acids have been calculated (Table III). These formulas have also been used to calculate the values of $(P_K - P_{K'})$ in certain *cis* and *trans* acids from distances estimated with structural models. The agreement is close (Table IV). Isomeric *cis* and *trans* acids are shown to have nearly identical intrinsic constants.

Formulas 19 to 24 are given for calculating the dissociation constants of *higher* polyvalent acids, and of bases and ampholytes, where the intrinsic constants of the individual groups are known, and when the distances between the charges in the groups may be estimated. Formulas 26 to 29 summarize the relations between the "dissociation" (K), the "intrinsic" (K_0), the "titration" (G) and the "characteristic" (K_c) constants of a substance, and also give the activity correction.

THE CONCENTRATION OF VITAMIN B. III.

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In our last publication (1) on the same subject we reported on a fraction which was potent in daily doses of 0.0001 gm. The yield of the product was about 3 to 5 per cent (in units) of the starting material which was prepared by a slightly modified Osborne and Wakeman process. Further progress of the work obviously depends on a successful reduction of the losses associated with the concentration.

A way has now been found which leads to material fully as potent and at times of even higher potency than the one previously described and which increases the yield in units from 3 to 5 per cent, as previously reported, to 35 to 50 per cent of the starting material. The new process, besides improving the yield, has the value of throwing a certain amount of light on the chemical properties of the vitamin.

The steps in the latest process are the following:

1. The Osborne-Wakeman fraction is extracted repeatedly with absolute alcohol until a dry non-hygrosopic powder is obtained. This is dissolved in water and the solution is brought to pH = 4.0. On standing a precipitate of inactive material is formed.

2. The active material obtained from the first step is deaminized by means of barium nitrite and sulfuric acid. The loss of material sustained in this step did not exceed 10 per cent.

3. From the product obtained by step (2), the active material is absorbed on silica gel and is extracted from it by the same process as described in the previous paper. The loss in this step in units was in general about 50 per cent and never exceeded 65 per cent.

The final product obtained is still a complex mixture, containing complex carbohydrates and nitrogenous substances and esters of phosphoric acid. It contains no amino nitrogen, but does contain sulfur, which is given off on treatment with alkali in the form of hydrogen sulfide. The sulfur content increases with purification,

but it is premature to pass an opinion as to whether the sulfur is a part of the active substance.

EXPERIMENTAL.

Purification of Osborne-Wakeman Fraction.—Material prepared according to Osborne and Wakeman and shaken with alcohol (cf. paper II) is dissolved in water and brought to a pH of 4.0. A considerable amount of insoluble material settles out after standing two days in the ice chest. This is removed by centrifuging. The remaining filtrate is active in 0.5 mgm. nitrogen per day, whereas the original O. W. fraction was active in 2 mgm. nitrogen. Both materials contain about 10 per cent nitrogen.

Deamination.—An amount of purified Osborne-Wakeman fraction corresponding to about 6 grams nitrogen is taken up in 200 cc. cold saturated barium nitrite solution. To the solution 10 per cent sulfuric acid is gradually added (butyl alcohol is used to prevent foaming) until the solution is free of barium. This strongly acid solution is then centrifuged, concentrated under diminished pressure to a volume of about 120 cc. and to it 500 cc. of absolute alcohol are added. The precipitate collected by centrifuging is dried. The filtrate is again concentrated to 10 cc. and precipitated by an excess of absolute alcohol.

Purified O. W. fraction		
2.94 grams N (0.5)		
[5900] units		
deaminized, precipitated in 80 per cent alcohol.		
Filtrate in 99 per cent alcohol		Precipitate
Filtrate	Precipitate	0.76 gram N (0.14)
1.7 grams N (inactive)	0.36 gram N (0.7)	[5400] units
[0] units	[500] units	Yield 90 per cent

Purified O. W. fraction	
4.8 grams N (0.65)	
[7400] units	
deaminized, precipitated in 80 per cent alcohol.	
Filtrate	Precipitate
3.0 grams N (5)	1.5 grams N (0.18)
[600] units	[8400] units
	Yield 100 per cent

Deamination product

Calculated for ash-free substance

Analysis: C = 38.2 per cent

H = 6.0 per cent

N = 10.3 per cent

P = 12.0 per cent¹

total S = 1.46 per cent

thiol S = 0.38 per cent (determined according to
Abel's Insulin Method)NH₂ = nil

This substance is a yellow powder which reduces Fehling's solution strongly after hydrolysis. It gives abundant PbS after heating with alkaline lead solution and also gives a faint nitroprusside reaction.

Expressed in the usual way (three-day period) the activity was about 0.15 mgm. nitrogen per day, equivalent to 1.4 mgm. substance. Experiments with feeding of this material over a period of two weeks showed that for normal growth during that time about 2 mgm. substance per day were needed.

Silica Gel Extraction.—This process was accomplished in the same way as described in paper II; absorption on the gel at pH 5, two preliminary extractions at pH 3 followed by one at pH 9.5. The silica gel was previously purified by repeated extractions with strong hydrochloric acid and nitric acid; hydriodic acid and lithium hydroxide were used throughout the extraction process to establish the required pH. The neutralized alkaline extraction product was concentrated to 200 cc., then filtered through a collodion membrane in

¹ Includes some inorganic phosphates.

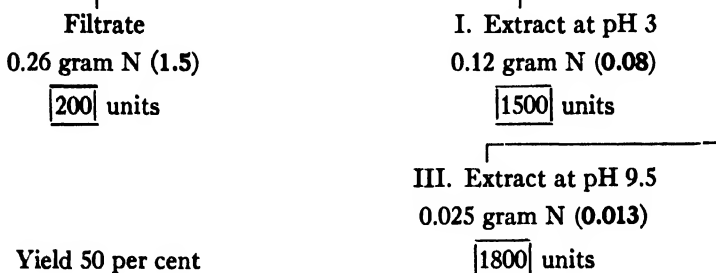
order to remove inorganic colloidal material (principally silicic acid), again concentrated to about 10 cc., precipitated by excess of dry acetone and washed.

Deaminized material

0.65 gram N (0.18)

3600 units

extracted with 900 grams purified silica gel.

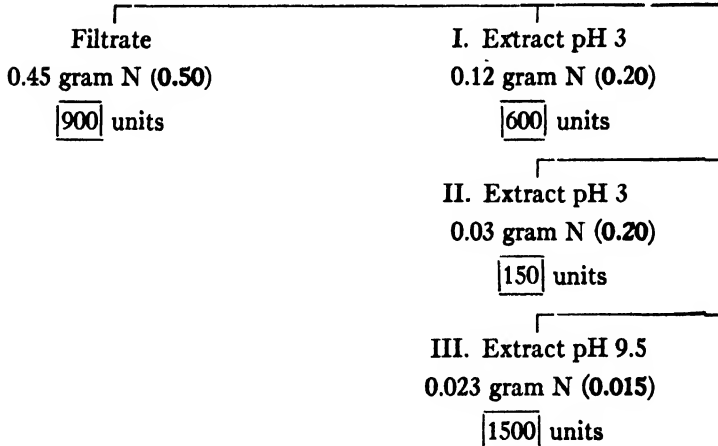


Deaminized material

0.7 gram N (0.15)

4600 units

extracted with 1000 grams silica gel.



Yield 35 per cent

Silica extraction product

Calculated for ash-free substance

Analysis: C = 41 per cent

H = 6.7 per cent

N = 9.0 per cent

P = 16.5 per cent²

total S = 3.9 per cent³

thiol S = 2.0 per cent

NH₂ = nil

The substance, which is a yellow powder, reduces Fehling's solution after hydrolysis; gives lead sulfide after heating with an alkaline lead solution and also gives a positive orcin test.

Its activity is approximately 0.01 mgm. nitrogen per day corresponding to about 0.1 mgm. substance. The best activity observed was 0.08 mgm. substance per day. Feeding over a fourteen-day period with 0.15 mgm. substance gave a normal growth curve.

REFERENCE.

- (1) LEVENE, P. A., AND VAN DER HOEVEN, B. J. C.: Jour. Biol. Chem., 1925, lxx, 483.

² Free from inorganic phosphates.

³ This includes a small proportion of inorganic sulfates present in the material.

THE ACTION OF HYDRAZINE HYDRATE ON URIDINE.

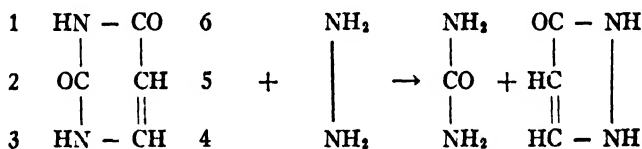
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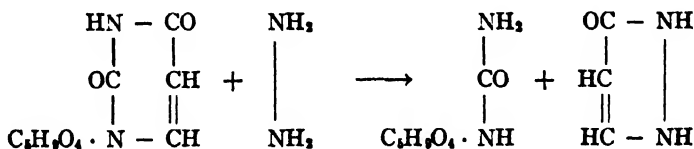
In previous communications¹ from this laboratory, evidence has been submitted supporting the view that in the pyrimidine nucleosides the sugar molecule is linked to nitrogen atom (3). We are now able to report in favor of this structure additional, more conclusive evidence based on an investigation of the action of hydrazine on uridine.

It has recently been shown² that hydrazine reacts with uracil to form urea and pyrazolone,



the two products of the reaction being isolated as xanthyl derivatives. Analogously, thymine yields urea and 4-methylpyrazolone.

An extension of this reaction to uridine should theoretically lead to definite evidence in regard to the position of the sugar linkage. If the sugar is in position (3), we may expect to obtain as primary products pyrazolone and urea riboside:



¹ Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608. Levene, P. A., *J. Biol. Chem.*, 1925, lxiii, 653. Levene, P. A., Bass, L. W., and Simms, H. S., *J. Biol. Chem.*, 1926, lxx, 229.

² Fosse, R., Hieulle, A., and Bass, L. W., *Compt. rend. Acad.*, 1924, clxxviii, 811.

On the other hand, if the sugar is linked to carbon atom (4), we may expect to obtain urea and the ribose derivative of pyrazolone. The products indicated in one or the other of these reactions should theoretically be formed, provided that the linkage between the sugar and the pyrimidine is not broken by the action of hydrazine.

If ribose were linked to carbon atom (4) of the pyrimidine, it is reasonable to assume *a priori* that the union would resist the action of hydrazine, since in this case the sugar would be united to the pyrimidine by a carbon to carbon bond.

It is difficult, however, to predict on theoretical grounds whether the sugar would be split off by the action of hydrazine if it were united to the pyrimidine in position (3). It was therefore necessary to test this possibility experimentally.

Fischer and Helferich³ and Levene and Sobotka⁴ were unsuccessful in their attempts to prepare pyrimidine glucosides with the sugar attached to a nitrogen atom. Since no suitable pyrimidine derivative was available, experiments were performed on urea glucoside, inasmuch as uracil may be regarded as a substituted urea. These experiments proved, first, that this glucoside is not hydrolyzed by hydrazine, since it is not possible to isolate urea from the reaction product by means of xanthyrol, whereas urea is readily isolated from a mixture of urea, glucose, and hydrazine. Second, it was shown that urea glucoside reacts with hydrazine to form a product which does not yield an insoluble xanthyl derivative. This conclusion was drawn from the following experimental results. When urea glucoside is treated with xanthyrol in acetic acid solution, it forms a characteristic monoxanthyl derivative (unsubstituted urea yields a dioxanthyl derivative). However, if urea glucoside is first warmed with hydrazine, neither xanthyl urea glucoside nor dioxanthylurea can be isolated from the reaction product.

On the basis of these considerations the following conclusions were formulated:

1. The isolation of a ribose-free pyrazolone derivative from the product of the reaction between uridine and hydrazine may be re-

³ Fischer, E., and Helferich, B., *Ber. chem. Ges.*, 1914, xlvii, 210. Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 1377.

⁴ Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1925, lxx, 469.

garded as an indication that ribose is linked to the pyrimidine in position (3).

2. The isolation of free urea may be regarded as evidence in favor of the linkage in position (4).

From this standpoint, the experiments on uridine afforded convincing evidence in favor of the structure which assumes a union of the sugar with nitrogen atom (3) of the pyrimidine. From the reaction product, ribose-free pyrazolone was isolated in the form of its dioxanthyl derivative. Furthermore, it was shown that free urea was not formed in appreciable quantity (if at all). Further efforts are being directed toward the isolation of the hydrazine derivative of urea riboside.

SUMMARY.

1. It has been shown by Fosse, Hieulle, and Bass that uracil reacts with hydrazine to form urea and pyrazolone.

2. Urea can be isolated by means of xanthidrol from a mixture containing urea, glucose, and hydrazine.

3. Urea glucoside forms a difficultly soluble monoxanthyl derivative.

4. From the reaction product of hydrazine and urea glucoside neither free urea nor urea glucoside can be isolated by means of xanthidrol.

5. From the reaction product of hydrazine and uridine a ribose-free derivative of pyrazolone, but no perceptible quantities of urea, can be isolated.

6. It is therefore concluded that no perceptible quantity of ribose is liberated from uridine by the action of hydrazine, and hence, that the isolation of ribose-free pyrazolone is an indication that in uridine the union of ribose to uracil is in position (3) of the pyrimidine. This conclusion is further supported by the failure to isolate an appreciable quantity of urea.

EXPERIMENTAL.

Action of Hydrazine on Uridine.

0.4 gm. of uridine was heated in a stoppered test-tube with 1.00 cc. of hydrazine hydrate⁵ at 65°C. for 1 hour. The reaction mixture,

⁵ Kahlbaum hydrazine hydrate for analysis.

which consisted of a turbid, light brown solution, was washed into a flask with 13.0 cc. of water and 14.0 cc. of glacial acetic acid. To this solution was added a solution of 1.4 gm. (> 4 mols)⁶ of xanthyrol in 14.0 cc. of glacial acetic acid. After standing overnight, the precipitate was filtered off by suction and was then washed with water, alcohol, and again with water. The dried product, after recrystallization from acetone, melted at 210–213°C. The recrystallized material, after drying at 100°C. under reduced pressure over sulfuric acid, gave an analysis corresponding to dioxanthylpyrazolone.

No. 520. 0.1018 gm. substance: 0.2910 gm. CO₂ and 0.0420 gm. H₂O.
 0.1455 " " : (Dumas) 7.80 cc. N₂ at 21°C. and 769 mm.
 C₁₂H₁₀O₂N₂. Calculated. C 78.38, H 4.54, N 6.30.
 Found. " 77.95, " 4.64, " 6.32.

In a series of experiments, each with 0.40 gm. of uridine, the precipitate obtained by means of xanthyrol weighed 0.50 to 0.70 gm. Assuming that the precipitate consisted entirely of dioxanthylpyrazolone, these values correspond to 0.09 to 0.13 gm. of pyrazolone,⁷ or 0.27 to 0.38 gm. of uridine.

Although urea is precipitated quantitatively⁸ by xanthyrol under these conditions, none of these precipitates appeared to contain dioxanthylurea, the strongest proof of this conclusion being their complete solubility in comparatively small volumes of acetone. In this connection it should be noted that in the work on uracil² the isolation of dioxanthylurea was accomplished by extracting the dioxanthylpyrazolone from the mixture by means of acetone.

To substantiate our conclusion that these two xanthyl derivatives could be separated by fractional solution in acetone, it was necessary to determine approximately their solubilities in this solvent. The solubility of dioxanthylpyrazolone is roughly 0.2 gm. per 100 cc. of acetone at the boiling point, while the solubility of dioxanthylurea is less than 0.008 gm. per 100 cc.

⁶ Four mols of xanthyrol were employed to insure complete precipitation of the possible products of the reaction; *i.e.*, urea and pyrazolone.

⁷ It is not known whether pyrazolone is precipitated quantitatively by xanthyrol.

⁸ Fosse, R., *Ann. Inst. Pasteur*, 1916, **xxx**, 525.

A second series of experiments with uridine was then performed according to the following procedure. 0.20 gm. of the nucleoside was heated with 1.00 cc. of hydrazine at 65°C. for 1 hour. The precipitation with 4 mols of xanthydrol was carried out as in the preceding experiments. The precipitates weighed 0.25 to 0.28 gm. (\equiv 0.048 to 0.053 gm. of pyrazolone \equiv 0.14 to 0.15 gm. of uridine), and were completely soluble in 100 to 125 cc. of acetone. Hence, if dixanthylurea were present, the quantity was less than 0.01 gm. (\equiv 0.001 gm. of urea \equiv 0.006 gm. of uridine).

Isolation of Urea from Reaction Mixtures Containing Hydrazine and a Sugar.

It has already been shown in the investigation of the action of hydrazine on uracil² that urea can be isolated from a reaction mixture containing pyrazolone and excess hydrazine. In order to comply with the conditions of our experiments, it was necessary to prove that urea can also be isolated from a reaction mixture containing hydrazine and a sugar.

0.1 gm. of urea was heated with 0.5 cc. of hydrazine at 65°C. for 1 hour. From the reaction mixture the urea was precipitated as dixanthylurea. After recrystallization from pyridine it melted at 263°C. For analysis it was dried at 100°C. under reduced pressure over sulfuric acid.

No. 515. 0.1000 gm. substance: 0.2830 gm. CO₂ and 0.0426 gm. H₂O.
 0.1610 " " : (Dumas) 9.50 cc. N₂ at 28°C. and 759 mm.
 C₁₇H₁₆O₂N₂. Calculated. C 77.14, H 4.81, N 6.66.
 Found. " 77.17, " 4.77, " 6.70.

A mixture of 0.1 gm. of urea and 0.3 gm. of glucose was heated with 0.5 cc. of hydrazine at 65°C. for 1 hour. The dixanthylurea was precipitated and purified as in the preceding experiment. It melted at 263°C. and gave the following analysis.

No. 516. 0.0979 gm. substance: 0.2786 gm. CO₂ and 0.0420 gm. H₂O.
 0.1562 " " : (Dumas) 9.00 cc. N₂ at 25°C. and 761 mm.
 C₁₇H₁₆O₂N₂. Calculated. C 77.14, H 4.81, N 6.66.
 Found. " 77.60, " 4.80, " 6.60.

A similar result was obtained when ribose was substituted for glucose.

Xanthyl Urea Glucoside.

Urea glucoside was prepared according to the methods of Schoorl⁹ and Helferich and Kosche.¹⁰

The xanthyl derivative was prepared by adding to a solution of 2.0 gm. of the ureide in 50.0 cc. of 50 per cent aqueous acetic acid a solution of 2.0 gm. (> 1 mol) of xanthydrol in 25.0 cc. of glacial acetic acid. The solution showed a yellow color for an instant, then became very viscous, and in a few minutes had become a heavy gel. This gel was very difficult to filter and attempts to purify the solid by recrystallization were unsuccessful. For purification it was washed repeatedly with alcohol on a suction filter. It was dried at 100°C. under reduced pressure over sulfuric acid.

No. 365. 0.0975 gm. substance: 0.2124 gm. CO₂ and 0.0516 gm. H₂O.
 0.1159 " " : (Dumas) 7.00 cc. N₂ at 23°C. and 748 mm.
 C₂₀H₂₂O₇N₂. Calculated. C 59.70, H 5.52, N 6.96.
 Found. " 59.40, " 5.92, " 6.85.

It melted with decomposition at 216–219°C.

The substance was found to have a low levorotation in saturated solution in acetic acid but no accurate reading could be obtained.

Action of Hydrazine on Urea Glucoside.

0.4 gm. of urea glucoside was heated with 0.50 cc. of hydrazine and 1.00 cc. of water at 65°C. for 1 hour. On cooling, the clear solution solidified. A test with xanthydrol showed that neither urea glucoside nor free urea was present.

⁹ Schoorl, N., *Rec. trav. chim. Pays-Bas*, 1903, xxii, 31.

¹⁰ Helferich, B., and Kosche, W., *Ber. chem. Ges.*, 1926, lix, 69.

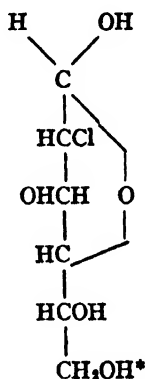
DEAMINATION OF 3-AMINOHEXOSES.

By P. A. LEVENE AND HARRY SOBOTKA.

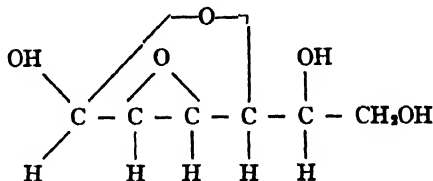
(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The present paper deals with the deamination of the so called epiglucosamine of Fischer, Bergmann, and Schotte.¹ This sugar is prepared from



in which the configuration of carbon atom (2) is not known. The formation of the amino sugar through the action of ammonia passes through the intermediate stage of an ethylene oxide,

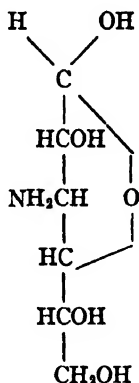


Hence, in the amino sugar, the configurations of carbon atoms (2)

¹ Fischer, E., Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, 1920, liii, 509.

* The ring structures of the sugars of this group are not yet known. The fact that the free epiglucosamine readily forms a second internal anhydride leads one to surmise that the original ring is five-membered.

and (3) are unknown. Recently Freudenberg, Burkhart, and Braun² prepared a new 3-aminohexose to which they assigned the following structure:



Inasmuch as this sugar was prepared from tolyl sulfodiacetone glucose, there is uncertainty of the configuration of carbon atom (3). The properties of the two amino sugars are distinctly different and the question arises whether these differences are due to dissimilarities in the configurations of only one carbon atom (2) or (3), or in those of both.

Dissimilarities in carbon atom (2) will disappear when the two sugars are converted into the corresponding osazones. Hence, if the osazones of the two sugars should show dissimilarities, the conclusion will follow that the configurations of carbon atoms (3) are not identical, and *vice versa*. The two osazones have been prepared, that of epiglucoamine by Levene and Meyer³ and that of the new amino sugar by Freudenberg and his coworkers. The two osazones were found somewhat different. The former had a sharp melting point at 207°C.; for the second only the decomposition point is given, which is at 207°C. Often the decomposition point lies much above the melting point. The specific rotation of the first was found -41° and that of the second -58° . If the two osazones are different, then one should have a configuration analogous to glucosazone and the other to that of allosazone. The latter two differ from one another in the following way: The glucosazone has the higher melting point and the higher

² Freudenberg, K., Burkhart, O., and Braun, E., *Ber. chem. Ges.*, 1926, lix, 714.

³ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1923, lv, 221.

levorotation. In the case of the two osazones of the amino sugars, the one with the higher rotation seems to have either the identical or a lower melting point than the isomer. It is to be regretted that Freudenberg and his coworkers, who had the two osazones in their hands, did not compare the melting points, inasmuch as the melting points of osazones depend so on the rate of heating. These authors, however, did compare their specific rotations and these may be considered correct. On the basis of the rotations, the osazone of Freudenberg and coworkers should be analogous to glucosazone and that of Levene and Meyer to allosazone. Such, indeed, was the conclusion of Freudenberg and coworkers, although they arrived at it on hypothetical grounds. However, the present assumptions may be regarded only as an indication for further and more rigorous investigation.

In the way of a beginning to such work, we decided to compare the osazones of the desamino sugars in the hope that the differences in the properties of these would be more pronounced than in the case of the amino sugars. Inasmuch as the preparation of the 3-amino sugars is very laborious, we are reporting for the present on the properties of one desamino sugar only, namely of the anhydrosugar derived from epiglucosamine, particularly since the observations on this substance have several points of fundamental importance.

Aminohexoses are now known with the amino group in position (2), (3), or (6). The first group on deamination gives rise to 2-5-anhydrosugars; the third, according to Freudenberg and Doser,⁴ gives rise in the main to the parent hexoses. The behavior in this respect of the 3-aminohexoses has not yet been established. We now find that epiglucosamine forms on deamination an anhydrosugar with the ring (as seen from the model) most probably in 3-6 position. One anhydrosugar of this type has already been prepared by Fischer and Zach⁵ by the action of baryta on triacetylmethylglucoside bromohydrin. In the case of Fischer and Zach the sugar had the configuration of an anhydroglucose, also probably with ring in position 3-6. The comparison of the osazone of the anhydrosugar of Fischer and Zach with that obtained from epiglucosamine should furnish certain

⁴ Freudenberg, K., and Doser, A., *Ber. chem. Ges.*, 1923, lvi, 1243.

⁵ Fischer, E., and Zach, K., *Ber. chem. Ges.*, 1912, xlv, 456.

indications as to the structure of the new anhydrosugar. Fischer and Zach record the melting point of their osazone at 180°C. and decomposition point at 200°C. Our osazone had a melting point of 160°C. and a decomposition point at 190°C. Fischer and Zach do not record the specific rotation of their osazone, hence the two osazones cannot be compared from that view-point. It is interesting, however, to note that glucosazone has a higher melting point than allosazone, and if there exists an analogy between the osazones of the simple sugars and those of the anhydrosugars, then the configuration of the new osazone should be that of 3-6 anhydroallosazone. For a final conclusion more evidence is required. Further work on the anhydrosugars is in progress. The results thus far obtained are published now in view of the fact that one of the authors had to discontinue his cooperation in this investigation.

EXPERIMENTAL.

The starting material was the acetate of 1-methyl-3-amino-hexose previously described by Levene and Meyer. The deaminized free sugar may be obtained either by deaminizing the glucoside and subsequently hydrolyzing the anhydroglucoside, or first hydrolyzing the aminoglucoside and subsequently deaminizing the amino sugar. Only the second method will be described here in detail inasmuch as this method proved by far the most economical from the view-point of yield.

2.0 gm. of epiglucosamine acetate were dissolved in 50 cc. of 2 per cent hydrochloric acid, and the solution was boiled for 2 hours. The solution possessed a reducing power equivalent to 0.6 gm. of glucose.

For deamination the solution was cooled to 0°C. and to it an equivalent of silver nitrite was added; after 8 hours $\frac{1}{2}$ of an equivalent of silver nitrite and the corresponding amount of hydrochloric acid were added. The final solution was freed from the excess of silver ions, and it then possessed a reducing power equivalent to 1.3 gm. of glucose. The solution was then neutralized and concentrated to a volume of 100 cc. A solution of 1.5 gm. of phenylhydrazine in glacial acetic acid was added to it and the combined solution was digested on the

water bath for 10 minutes. A very voluminous, gelatinous, orange-yellow precipitate formed which consisted of microscopic rosettes. To the filtrate from the first crop of the osazone was added a second portion of 1.0 gm. of phenylhydrazine dissolved in glacial acetic acid. The solution was allowed to digest 10 minutes and the second crop of osazone, which had the same appearance as the first, was filtered off and dried.

The two crops of osazone were combined and recrystallized from water containing a little pyridine. The material prepared in this manner, on heating in a capillary tube, contracted at 120°C., melted at 160°C., and decomposed at 190°C. The recrystallization from pyridine and water was repeated and the product behaved on heating in the same manner as before recrystallization. The osazone was then recrystallized from dilute alcohol, from which solvent it deposited in bright yellow microscopic rosettes. It melted sharply at 160°C. and decomposed at 185–190°C.

0.0456 gm. substance: 0.1040 gm. CO₂ and 0.0242 gm. H₂O.

0.0516 " " : 7.40 cc. N₂ at 25°C. and 764.0 mm.

C₁₈H₂₀O₈N₄ (340.3). Calculated. C 63.49, H 5.93, N 16.47.

Found. " 63.50, " 6.11, " 16.51.

Two samples were used for the measurement of the optical activity. One sample was obtained by recrystallization from pyridine and water. It rotated in methyl alcoholic solution as follows:

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{0.500 \times 1} = -44^\circ. \quad [\alpha]_D^{20} = \frac{\text{Equilibrium.}}{0.500 \times 1} = -8^\circ.$$

The second sample was obtained by further recrystallization of the first sample from dilute alcohol. Its rotation in methyl alcoholic solution was the following.

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{0.500 \times 1} = -24^\circ. \quad [\alpha]_D^{20} = \frac{\text{Equilibrium.}}{0.500 \times 1} = -8^\circ.$$

NOTE ON THE ACTION OF AMMONIA ON PROPYLENE OXIDE.

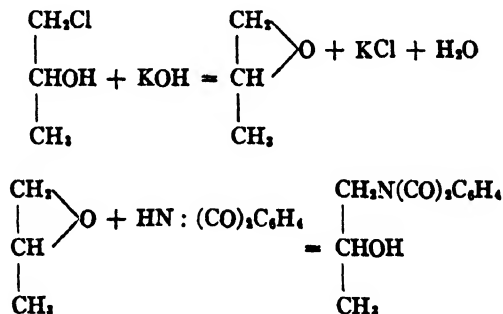
BY P. A. LEVENE AND A. WALTJ.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 1, 1926.)

In a previous communication on the configurational relationships of lactic and 3-hydroxybutyric acids,¹ a method was given for the preparation of aminohydroxypropane by the action of ammonia on propylene oxide. It is generally assumed that under such conditions the amino group enters position (1) and the hydroxyl position (2). The reason for the assumption is the following: It had been found that 1-amino-2-hydroxypropane was formed by the action of potassium phthalimide on either 1-chloro-2-hydroxypropane or on 1-hydroxy-2-chloropropane. This result can be explained on the assumption that the first step of the reaction consists of the formation of propylene oxide which is subsequently ammonolized to form 1-amino-2-hydroxypropane.²

The set of reactions leading to the formation of the base, then, may be expressed as follows:



¹ Levene, P. A., and Waltj, A., *J. Biol. Chem.*, 1926, lxxviii, 415.

² Gabriel, S., *Ber. chem. Ges.*, 1916, xlix, 2120. Gabriel, S., and Ohle, H., *Ber. chem. Ges.*, 1917, l, 804.

Thus, *a priori*, it is to be expected that the amine prepared by the action of ammonia directly on the oxide also has the composition of 1-amino-2-hydroxypropane. This amine and its isomer can be easily differentiated from each other by the melting points of the two isomeric hydroxypropylphenylthioureas derived from them. The derivative of 1-amino-2-hydroxypropane has a melting point of 108–109°C., whereas that of the isomer is at 141°C. The substance prepared by us melts at 107–108°C., thus showing the correctness of the assumption that on addition of ammonia to propylene oxide 1-amino-2-hydroxypropane is formed.

Incidentally it may be added that under the conditions adopted by us the formation of secondary and tertiary bases is reduced to a minimum.

EXPERIMENTAL.

Propylene Oxide.—A double neck distilling flask containing 65 gm. of potassium hydroxide dissolved in 85 cc. of water was immersed in a water bath. The flask, which held a dropping funnel containing 95 gm. of propylene chlorohydrin, was connected with a condenser through which water at 0°C. was circulated. The temperature of the water bath was then raised to 50°C. When this temperature was reached, one-third of the chlorohydrin was added to the alkaline solution and the temperature was quickly raised to 60°C. When the condensation of the propylene oxide became slow another one-third of the chlorohydrin was added, and finally the rest was added. The flask was vigorously shaken to drive over the propylene oxide. 49 gm., or 84 per cent of the theoretical yield, of propylene oxide were thus obtained.

Aminoisopropyl Alcohol.—15 gm. of the propylene oxide described above were poured into 2 liters of concentrated aqueous ammonia at 0°C. and allowed to stand in a refrigerator overnight. The solution was then concentrated under reduced pressure at room temperature until the odor of ammonia disappeared. The residue, about 25 cc., was taken up in 60 cc. of absolute alcohol. This solution was cooled to 0°C. and anhydrous potassium carbonate was added and well mixed. The dry alcoholic solution was decanted and the remaining potassium carbonate paste was extracted several times with absolute

alcohol, adding fresh portions of potassium carbonate each time. The combined alcoholic extracts stood for a few hours over anhydrous potassium carbonate. The alcohol was then removed under reduced pressure and the aminoisopropyl alcohol distilled at 156–162°C. at 761 mm. The yield was about 70 per cent. It analyzed as follows:

0.0834 gm. substance: (Van Slyke) 5.67 cc. N₂ at 25°C. and 764.9 mm.

C_8H_9ON . Calculated. N 18.66.

Found. " 18.96.

In the distilling flask there remained a little of a higher boiling residue which crystallized on standing and apparently represented di-(hydroxypropyl) amine, or tri-(hydroxypropyl) amine, or a mixture of both.

If the extraction with absolute alcohol and anhydrous potassium carbonate is omitted and the residue, after the ammonia has been removed by distillation under diminished pressure, is directly distilled at ordinary pressure, the temperature rises constantly from 100–160°C. or above, so that only a very small amount of the amine boiling correctly can be obtained.

$$2\text{-Hydroxypropylphenylthiourea. } \text{CH}_3\text{CHOHCH}_2\text{NHC} \begin{array}{c} = \text{S.—To} \\ | \\ \text{NHC}_6\text{H}_5 \end{array}$$

2 gm. of aminoisopropyl alcohol were added 3.7 gm. of phenylisothiocyanate, the addition being accompanied by the evolution of heat. The substance thus obtained was recrystallized four times from water. It melted at 107–108°C. and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 9.48 cc. 0.1 N HCl.

$C_{10}H_{14}ON_2S$. Calculated. N 13.33.

Found. " 13.12.

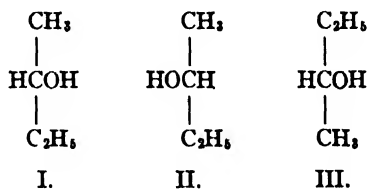
THE CONFIGURATIONAL RELATIONSHIP OF DEXTRO-METHYLETHYL CARBINOL TO DEXTRO-LACTIC ACID.

BY P. A. LEVENE, A. WALTI, AND H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 1, 1926.)

The configurations of aliphatic substances containing in their molecules several secondary alcoholic groups, or one or more secondary alcoholic groups in addition to a polar radicle as $-\text{COOH}$, $-\text{CHO}$, or $=\text{C}=\text{O}$, have been correlated to a single reference substance, lactic acid. Nothing is known, however, regarding the stereochemical relationships of simple secondary alcohols to the same substance of reference, nor is there any definite knowledge as to the stereochemical relationships of individual secondary alcohols among themselves. The case of secondary alcohols is complicated by the following fact. The first member of the series is methylethyl carbinol (I). Its

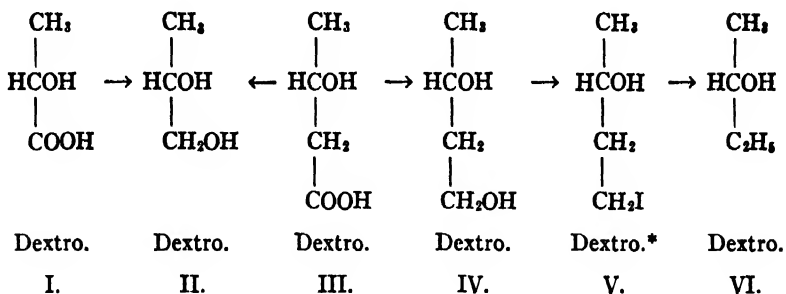


enantiomorph (II) may be represented also as ethylmethyl carbinol (III). A question has been raised as to whether or not the higher homologues of the methyl and ethyl series should rotate in opposite directions as their first members do. Two conflicting answers have been given to the question, both based on indirect evidence.¹

In the present communication data are given which permit the correlation of the configuration of dextro-methylethyl carbinol with

¹ Clough, G. W., *Proc. Chem. Soc.*, 1913, xxix, 357. Kenyon, J., *J. Chem. Soc.*, 1914, cv, 2226.

that of dextro-lactic acid. The arguments are presented in the following figures:



The reactions leading to the correlations of (I) to (IV) were described in previous communications by Levene and Haller² and Levene and Walti.³ The remaining reactions are described below. Thus dextro-methylethyl carbinol is correlated to dextro-lactic acid, which belongs to the *l* series; hence, dextro-ethylmethyl carbinol also belongs to the *l* series.

EXPERIMENTAL.

Preparation of Dextro-1, 3-Dihydroxybutane.—Dextro-1, 3-dihydroxybutane was obtained by reducing freshly prepared aldol with brewer's yeast according to the procedure of Neuberg and Kerb.⁴ It has been found unnecessary to distil the aldol after removal of the ether under reduced pressure. The glycol showed a rotation of $\alpha = +9.9^\circ$ in a 1 dm. tube.

The glycol obtained by the use of brewer's yeast had a specific rotation higher than that of the product obtained when baker's yeast was employed. A glycol obtained with baker's yeast gave $\alpha_D^{20} = +1.6^\circ$ to $+2.5^\circ$ in a 1 dm. tube, whereas the glycol obtained with brewer's yeast gave $\alpha_D^{20} = +9.9^\circ$ in a 1 dm. tube.

Diurethane of Dextro-1, 3-Dihydroxybutane.—To 0.9 gm. of 1, 3-

* The rotation of the iodohydrin could not be measured because of the dark color of the solution. The bromo derivative, however, was dextrorotatory.

² Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxxv, 49; 1926, lxxvii, 329; 1926, lxxix, 165.

³ Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1926, lxxviii, 415.

⁴ Neuberg, C., and Kerb, E., *Biochem. Z.*, 1918, xcii, 96.

dihydroxybutane were added 2.5 gm. of phenylisocyanate and the mixture was heated at 100° for 1 hour. The solid product was washed with petroleum ether, and was recrystallized three times from dilute alcohol. The diphenylurethane melted at 127–129°C. and analyzed as follows:

0.0500 gm. substance: (Kjeldahl) 3.10 cc. 0.1 N HCl.
 $C_{18}H_{20}O_4N_2$. Calculated. N 8.53.
 Found. " 8.68.

It had the following rotation in absolute alcohol.

$$[\alpha]_D^{20} = \frac{+3.45^\circ \times 100}{1 \times 6.48} = +53.24^\circ.$$

Preparation of 1-Iodo-3-Hydroxybutane.—Into 40 gm. of dextro-1,3-dihydroxybutane 57 gm. of dry hydrogen iodide (1 equivalent) were passed, the temperature being maintained at 0°C. The dark solution was heated in a water bath for $\frac{1}{2}$ hour. After cooling, 100 cc. of chloroform, 10 gm. of ice, and 5 cc. of water were added and the mixture was made neutral to Congo red with sodium carbonate. The chloroform was separated and the extraction with chloroform was repeated twice. The combined chloroform extracts were dried over sodium sulfate. The chloroform was removed by distillation under reduced pressure and the 1-iodo-3-hydroxybutane was distilled. It boiled at 58–60°C., $p = 0.4$ to 0.5 mm. The yield was 39 gm. The substance analyzed as follows:

0.1031 gm. substance: 0.1236 gm. AgI.
 C_4H_9OI . Calculated. I 63.50.
 Found. " 64.79.

Owing to the dark color of the solution it was not possible to measure its optical rotation.

Preparation of Dextro-1-Bromo-3-Hydroxybutane.—This product was obtained by passing 1 molecule of dry hydrogen bromide into 1 molecule of dextro-1, 3-dihydroxybutane ($\alpha = +9.9^\circ$) at 0°C. The acid mixture was neutralized and worked up in the same way as the above mentioned 1-iodo-3-hydroxybutane. The dextro-1-bromo-3-hydroxybutane boiled at 72–76°C. at 20 mm. and had a rotation of $\alpha_D^{20} = +2.53^\circ$ in a 1 dm. tube.

It analyzed as follows:

0.1413 gm. substance: 0.1740 gm. AgBr.

C_4H_9OBr . Calculated. Br 52.3.

Found. " 52.4.

Reduction of 1-Iodo-3-Hydroxybutane to Dextro-Methylethyl Carbinol.—60 cc. of a 10 per cent aqueous sodium hydroxide solution and 19 gm. of 1-iodo-3-hydroxybutane were placed in a shaking flask and the flask was then connected with a hydrogen cylinder. After replacing the air in the flask by hydrogen, 0.1 gm. of colloidal palladium in a little water was added. The absorption of hydrogen was rapid at first. The reduction was continued for 48 hours. Two 0.1 gm. portions of colloidal palladium were added during the course of the reaction. The reaction mixture was extracted with ether in a continuous extraction apparatus. The ether extract was carefully dried with sodium sulfate and then with 0.1 gm. of metallic sodium. The ether was distilled off, employing a fractionating column. From the residue of two such experiments the following fractions were obtained by fractional distillation.

95–99°C. = 1.5 cc.

99–104 " = 3.5 " $\alpha_D^{20} = +3.54^\circ$ in 1 dm. tube.

104–195 " = 2.5 " $\alpha_D^{20} = +2.69^\circ$ " 1 " "

The residue from the distillation was about 0.3 cc.

Phenylurethane of Methylethyl Carbinol.—To 0.74 gm. of the fraction boiling from 99–104°, 1.4 gm. of phenylisocyanate were added. The mixture was heated at 100–120° for 4 hours. Crystallization took place on standing overnight. The product was recrystallized three times from dilute alcohol. It melted at 62°C. The rotation of the substance in absolute alcohol was

$$[\alpha]_D^{20} = \frac{+0.81^\circ \times 100}{1 \times 10} = +8.1^\circ.$$

The substance analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5.40 cc. 0.1 N HCl.

$C_{11}H_{15}O_2N$. Calculated. N 7.25.

Found. " 7.56.

The above results were further confirmed by converting levo-methylethyl carbinol into its phenylurethane. The levo-methylethyl carbinol was obtained by resolution of the inactive alcohol following the procedure of Pickard and Kenyon.⁵

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{-1.12^\circ \times 100}{1 \times 12.85} = -8.71^\circ.$$

The phenylurethane was prepared in the usual way. Several recrystallizations from 75 per cent alcohol gave a product which melted at 64–65°C. and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5.26 cc. 0.1 N HCl.
C11H14O2N. Calculated. N 7.25.
Found. " 7.28.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{-0.88^\circ \times 100}{1 \times 5.25} = -16.76^\circ.$$

⁵ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 64.

LACTONE FORMATION OF LACTO- AND MALTOBIONIC ACIDS AND ITS BEARING ON THE STRUCTURE OF LACTOSE AND MALTOSE.

BY P. A. LEVENE AND HARRY SOBOTKA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 23, 1926.)

The knowledge of the structure of disaccharides was considerably advanced recently, first by the improvement in the use of methylation methods and in the handling of the methylated sugars, and furthermore by the introduction by Zemlen of the method of gradual degradation.

The method of methylation as well as the method of Zemlen is very laborious and requires considerable quantities of material. Therefore, any simple method yielding information as to the point of union of the two sugars should be found useful.

Recently Levene and Simms¹ have shown that sugar acids in which positions (4) and (5) are not substituted form in aqueous solution two lactones. These differ in the rates of formation and in their concentrations at the points of equilibrium. The six-membered ring is formed rapidly and at the point of equilibrium the lactone has a concentration of 20 to 30 per cent of the original acid; the five-membered ring is formed slowly and at the point of equilibrium the lactone has a concentration of 75 to 80 per cent. Thus it ought to be possible from observations on lactone formation to judge as to the part positions (4) and (5) play in the union between the components of a given disaccharide.

These considerations led us to investigate the lactone formation of two acids, namely lactobionic and maltobionic acids. The lactone formation was followed by observations on the changes in optical rotation and by titration. It must be recalled at this time that the

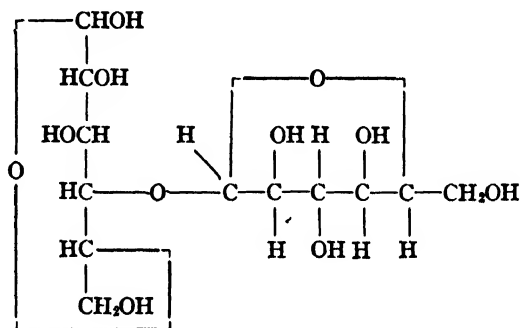
¹ Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1926, lxviii, 737.

velocity of hydrolysis of the $<1, 5>$ lactones is so rapid that it is not possible to detect their presence by titration.

The results of our observations are the following.

Maltobionic acid showed a slight rise in rotation which reached its maximum after 2 hours. The neutralization value remained constant throughout the experiment, which lasted 24 hours, and corresponded accurately to maltobionic acid, namely to 377.

Thus the conclusion is warranted that maltobionic acid forms a six-membered lactone. This conclusion leads to the assumption of a union in position (4) as follows:



This is one of the two possible structures formulated simultaneously by Irvine and Black² and by Cooper, Haworth, and Peat.³

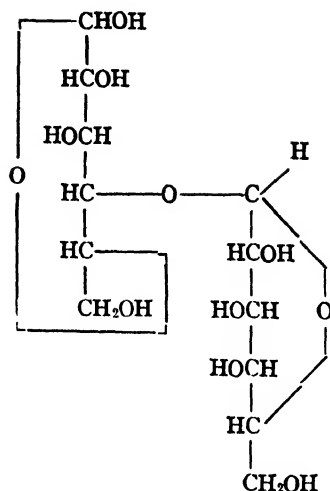
Lactobionic acid in aqueous solution showed a small but definite mutarotation and on titration gave a constant value throughout the experiment, which lasted 24 hours. Again in this case a $<1,4>$ lactone was not formed. This observation was to be expected on the basis of the work of Haworth and Leitch⁴ and of Zemplen⁵ who assign to lactose the following structure.

² Irvine, J. C., and Black, I. M. A., *J. Chem. Soc.*, 1926, cxxix, 862.

³ Cooper, C. J. A., Haworth, W. N., and Peat, S., *J. Chem. Soc.*, 1926, cxxix, 876.

⁴ Haworth, W. N., and Leitch, G. C., *J. Chem. Soc.*, 1918, cxiii, 188.

⁵ Zemplen, G., *Ber. chem. Ges.*, 1926, lix, 2402.



EXPERIMENTAL.

Mutarotation of Maltobionic Acid.—The calcium salt of maltobionic acid was used for the experiment. A convenient way of purifying the calcium salt of maltobionic and lactobionic acids is the following. To a 25 per cent solution of the bionic acid were added $1\frac{1}{2}$ volumes of 95 per cent alcohol. The calcium salt settled out as a sticky semifluid mass. The supernatant fluid was decanted, the residue was dissolved in a minimum of water, and the solution was poured gently with stirring into a large excess of methyl alcohol. Two or three reprecipitations sufficed to obtain a calcium salt having the composition required for the salt of a bionic acid.⁶

Maltobionic Acid.—1.0 gm. of the air-dried calcium salt containing 6 per cent moisture was dissolved in 20 cc. of water containing exactly 1 equivalent of hydrochloric acid. 1 cc. of this solution re-

⁶ We wish to acknowledge our indebtedness to Dr. Walther F. Goebel for a sample of maltobionic acid.

quired for neutralization 1.25 cc. of 0.1 N alkali, thus giving 377 as the equivalent weight of the maltobionic acid, which is exactly the value required by theory. The titration values and the changes in rotation are given in Tables I and II.

TABLE I.

Titration of 1 Cc. of a 0.125 N Solution of Maltobionic Acid with 0.1 N Alkali.

Time.	0.1 N alkali.
<i>hrs.</i>	<i>cc.</i>
0	1.25
2	1.24
3	1.25
4	1.25
21	1.25
23	1.25
26	1.25
29	1.25
31	1.25

TABLE II.

Changes in Rotation of the Above Solution.

$l = 2 \text{ dm.}$ $t = 24^\circ\text{C.}$ $\lambda = 5892 \text{ \AA.}$

Time.	α^d
<i>hrs.</i>	
0	9.58
1	10.69
2	10.72
3	10.73
4	10.74
6	10.73
23	10.74

* Accuracy of instrument = $\pm 0.02^\circ$.

Lactobionic Acid.—1.0 gm. of the air-dried calcium salt containing 0.8864 gm. of the anhydrous substance was dissolved in 20 cc. of water containing exactly 1 equivalent of hydrochloric acid. 1 cc. of this solution required for neutralization 1.17 cc. of 0.1 N alkali, thus giving for the equivalent weight of the salt the value of 377, which is

exactly that required by theory. The solution was then used for titrations and for the observations on changes of optical rotation as a function of time. The results are recorded in Tables III and IV.

TABLE III.

Titration of 1 Cc. of a 0.117 N Solution of Lactobionic Acid with 0.1 N Alkali.

Time.	0.1 N alkali.
<i>hrs.</i>	<i>cc.</i>
0	1.17
1	1.18
2	1.18
4	1.18
6	1.18
21	1.18
23	1.18
26	1.19
28.5	1.18
30	1.18

TABLE IV.

Changes in Rotation of the Above Solution. Two Experiments Recorded.

$l = 2$ dm. $t = 24^{\circ}\text{C}$. $\lambda = 5892 \text{ \AA}$.

Time.	α^*	
	Experiment I.	Experiment II.
<i>hrs.</i>		
0	1.62	1.66
0.25	1.75	
0.50	1.85	
1	1.96	1.92
2	2.02	2.03
3	2.02	2.03
4		2.06
5	2.03	
6		2.04
7	2.03	
8		2.05
24	2.03	2.06

* Accuracy of instrument = $\pm 0.02^{\circ}$.

THE FORMAL IDENTITY OF LANGMUIR'S ADSORPTION EQUATION WITH THE LAW OF MASS ACTION.

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In his work on the adsorption of gases on the surfaces of solids, Langmuir¹ deduced, on the basis of his theory, a number of equations relating the amount of gas adsorbed to the pressure at equilibrium. The simplest of these equations is

$$q = abp/(1 + ap) \quad (1)$$

where q is the amount adsorbed, p the pressure at equilibrium and a and b are constants. In connection with certain experiments² that were found to be fitted by such an equation, the writer noticed that an equation of exactly similar form could be derived from the law of mass action. Consider a reversible reaction between two substances A and B to form a compound AB. From the mass law,

$$\frac{(A)(B)}{(AB)} = k; \frac{(A)}{(AB)} = \frac{k}{(B)}; \frac{(A) + (AB)}{(AB)} = \frac{k + (B)}{(B)}; (AB) = \frac{\frac{(A) + (AB)}{k}(B)}{1 + \frac{(B)}{k}}. \quad (2)$$

For an experiment in which the total A (that is, the sum of the concentrations of A and AB) is kept constant, Equation 2 is of the same form as Equation 1. The variable AB and B correspond to q and p , while the constant $1/k$ corresponds to a and the constant $A + AB$ to b . Hence it must be concluded that the agreement of experimental data with an equation of this type is not decisive in determining whether the reaction in question is one of adsorption or chemical combination.

¹ Langmuir, *J. Am. Chem. Soc.*, **40**, 1384 (1918).

² Hitchcock, *J. Gen. Physiol.*, **8**, 61 (1925-26).

This fact seems to have been overlooked by H. Rinde,³ who found that certain data of Jacques Loeb⁴ on the equilibrium between gelatin solutions and hydrochloric acid could be fitted by such an equation. Rinde concluded that "it is therefore very likely that the 'reaction' between gelatin and HCl is not a chemical reaction in the sense assumed by Loeb, but an adsorption process. . . ." Obviously Rinde's calculation adds nothing to the decision of this question. Other data bearing on the case of gelatin and hydrochloric acid have been presented by Jacques Loeb⁵ and by the writer.⁶

³ Rinde, *Phil. Mag.*, [7] 1, 46 (1926).

⁴ Loeb, "Proteins and the Theory of Colloidal Behavior," McGraw-Hill Book Co., New York and London, 2nd ed., 1924, p. 183.

⁵ Ref. 4, Chapters 2 and 4.

⁶ Hitchcock, *J. Gen. Physiol.*, 6, 95, 201 (1922-23).

THE PENETRATION OF BASIC DYE INTO NITELLA AND VALONIA IN THE PRESENCE OF CERTAIN ACIDS, BUFFER MIXTURES, AND SALTS.

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(Accepted for publication, October 7, 1926.)

I.

INTRODUCTION.

It has been shown that the pH value of the cell sap plays¹ an important rôle in the accumulation of the basic dye, brilliant cresyl blue, in the living cell of *Nitella*, and in view of this it is important to study the changes in the rate of penetration produced by varying the pH value of the sap. Experiments of this sort, made by McCutcheon and Lucke,² and by the writer,³ showed that the penetration of ammonia increases the pH value of the sap and decreases the rate of penetration of the dye.

The present paper deals with experiments on the rate of penetration of the dye in presence of acids and buffer mixtures. These experiments are of interest in connection with the hypothesis⁴ that brilliant cresyl blue exists in aqueous solution in two forms, called for convenience DB and DS. DB, the form which predominates at higher pH values, represents a free base while DS exists predominantly at lower pH values and is a dissociated salt. A normal living cell of *Nitella* is assumed to be chiefly permeable to DB and only very slightly permeable to DS. The present problem is to find the nature of the factors controlling the penetration of DB.

¹ Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 561.

² McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923-24, vi, 501.

³ Irwin, M., *J. Gen. Physiol.*, 1925-26, viii, 147.

⁴ Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 75.

II.

Methods.

Only general methods will be given here: special methods for each set of experiments will be described in connection with the results.

The experiments were carried out in an incubator at $25^{\circ}\text{C.} \pm 0.5^{\circ}$ having air holes through which diffused light entered.

Only living cells were used. In order to obtain cells in the same condition for experimentation uniformity as to length, thickness, and external appearance was attended to. In the case of *Nitella flexilis* the cells used were taken from the central portion of the plant, midway between the tip and the root. Seasonal changes bring about differences in the permeability of the cells, so that a series of comparative experiments were made on the same lot of cells collected within a short period (near New York in spring unless otherwise stated).

A control experiment was always carried out by removing cells directly from tap water and placing them in the same dye solution as in the case of the test experiment (in which the cells were given some special treatment before being placed in the dye). The rate of penetration obtained from the control experiment was used as a standard of comparison in order to determine the change in the rate of penetration of the dye caused by varying the media in which the cells were placed previous to exposure to the dye solution.

Every determination given represents an average of over 60 experiments and the probable error of the mean is in all cases less than 7 per cent of the mean.

In the case of *Valonia macrophysa* (collected in Bermuda), the procedure was as follows: The clusters of cells were pulled apart and the individual cells were allowed to stand in pans of sea water (which was changed daily) for over 2 weeks in the laboratory (exposed during the day to diffused light). During this period the cell wall at the point of detachment thickened somewhat. This precaution was taken to diminish irregularity in the rate of penetration and the susceptibility of cells to injury upon exposure to solutions.

Small cells (each having a volume of about 0.1 cc.) with one point of detachment, and having practically no attached cells, were chosen. Care was taken to remove adhering organisms or deposits from the surface of the cell.

The detection of an early stage of reversible injury is a very difficult matter, especially with *Valonia*. In the case of *Nitella* an increase in the rate of accumulation of the dye may serve as an indication of a preliminary stage of an injury under certain conditions but this does not seem to be markedly evident in *Valonia*. The exit of halides from the vacuole of *Nitella* or the entrance^{5,6} of SO_4 into *Valonia* seems to indicate advanced stages of injury.

⁵ Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-26, viii, 131.

Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, ix, 255.

⁶ Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

Cells in good condition are turgid: as they become injured the turgidity diminishes. An experienced experimenter can, to a certain extent, predict the degree of resistance of the cells of *Nitella* and *Valonia* to experimental treatment by the turgidity as ascertained by touching them. This method was used by the writer as a rough guide to the condition of the cells but it does not serve to tell whether injury is reversible. The criterion of irreversibility of injury employed by the writer was as follows: cells which had been exposed to experimental conditions were replaced in the normal medium (*Nitella* in tap water, *Valonia* in sea water), and at intervals during 2 days the rate of mortality was compared with that of the control cells (without exposure to experimental conditions). The criterion of death for *Nitella* was a complete and permanent loss of turgidity, and for *Valonia* either a complete collapse of the cell or disarrangement of chlorophyll and its appearance in the vacuole, so that the greater part of the cell surface appeared colorless. Another test of the condition of the cells is to observe the length of time it takes for them to die in the experimental solutions.

It is not possible, however, to determine experimentally whether the cell was injured at the time of experiment, unless the injury happened to be irreversible. By using these tests an attempt was made to keep the cells uniform during the experiments.

The dye used was made by Grüber and was dissolved in buffer solutions (M/150) in the case of *Nitella* and in sea water in the case of *Valonia*. The pH values of the solutions determined colorimetrically were checked as much as possible by means of the hydrogen electrode. Solutions were not stirred unless otherwise stated.

The determination of the concentration of the dye in the sap was made colorimetrically. With *Nitella*, the cell was gently wiped and was cut at one end, so that the sap could be squeezed out onto a glass slide. With *Valonia* the surface of the cell was punctured with a sharp capillary tube and the sap was drawn up from the vacuole into the tube, from which it was pushed out onto a glass slide. In both cases the sap was drawn up into capillary tubes and the color was matched with capillary tubes of the same diameter containing standard dye solutions.

To determine the pH value of the sap a definite volume was taken by filling a tube for 2 inches with the sap. Indicator solution was drawn up into another tube for a distance of $\frac{1}{8}$ of an inch. The contents of both these tubes were pushed out onto a glass slide and thoroughly mixed. This mixture was then drawn up into a capillary tube and the color matched with that of the capillary tube containing a mixture of standard buffer solution at a known pH value and the same amount of the indicator (the mixture was prepared in the same manner as in the case of the sap). Care was taken to have the least possible contamination of the sap by CO₂ from the breath of the experimenter, as well as to prevent escape of CO₂ into the air, as far as possible.

The color of the indicators changed on standing in a buffer solution containing artificial *Valonia* sap, and also on standing in the natural expressed sap, but the color of the indicators did not change during the time required to determine the pH value of the sap.

The salts in the sap of *Nitella* (about 0.1 M halides) do not seem to affect the indicator seriously, but those in the sap of *Valonia* (about 0.6 M halides) have a very definite effect. In view of the fact that we know so very little about the salt error in general, and possible specific effects of individual salts on these indicators, it will be necessary to study this question carefully before absolute pH values of the sap of *Valonia* can be given.

Another possible source of error in the case of *Valonia* is that the sap is so little buffered that an addition of indicator solution may bring about a change in the pH value of the sap; careful experiments must therefore be made to avoid this error. On the other hand, the sap of the *Nitella* used by the writer is buffered so that this source of error may be negligible. Since only approximate and relative values are desired the pH values of the sap of *Valonia* and *Nitella* given in this paper represent values without a correction for salt error, determined by means of one concentration of indicator dissolved in distilled water of pH 5.8 (approximately the pH value of the sap), or indicator dissolved in alcohol (methyl red). For one series of changes in the pH values only one indicator is used. For example, when experiments were made by exposing cells to a solution of NH_4Cl , brom-cresol purple was used: in the case of cells placed in acid solutions methyl red was used. Brom-cresol green was used to check the values obtained with methyl red, but in view of the fact that the color above pH 5.2 was not satisfactorily matched, only a very rough estimation of the pH value of the normal sap could be made by this indicator. Each indicator is taken from the same stock solution for each series of experiments.

III.

The Decrease in the Rate of Penetration of Dye When the pH Value of the Sap Is Lowered by Entrance of Acetic Acid.

The cells were divided into four lots. One lot was placed in an acetate buffer mixture at pH 5.1, and at the end of 10 minutes the pH value of the sap was compared colorimetrically with that of the normal cell sap. It was found to have decreased⁷ from pH 5.5 (normal) to pH 4.9.

⁷ It may be added here that these experiments show that acetic acid enters the vacuole rather easily from an acetate buffer mixture and decreases the pH value of the sap until the internal pH value is less than the external. This agrees with the results obtained by many investigators showing that weak acids enter the living cells. The writer's experiments also show that the pH value of the sap may be raised again when acetic acid is allowed to come out of the vacuole by placing the cells in a solution containing no acetic acid (the more alkaline the external pH value, the more rapid is the rate of exit of acetic acid from the vacuole).

The second lot of cells was placed in the acetate buffer mixture at pH 5.1, and after 10 minutes they were removed, wiped, rinsed for 5 seconds in phosphate buffer mixture at pH 6.6, wiped, and placed in fresh phosphate buffer mixture at pH 6.6. After 1 minute the cells were removed, and the pH of the sap was determined. It was found to be pH 5.2, which is 0.3 pH lower than that of the normal cell sap.

TABLE I.

Comparison of the amount of brilliant cresyl blue in the vacuole when the living cells of *Nitella* are placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after previous exposure to M/150 acetate buffer solution at pH 5.1 for different lengths of time. The rate of penetration of dye in the case of cells directly transferred from the tap water to the dye solution is used as the standard of comparison.

External solutions.		In tap water at pH 7.7.	In acetate buffer solution 5 sec.	In acetate buffer solution 1 min.	In acetate buffer solution 10 min.
When dye solution is not stirred or changed.	Amount of dye in sap.....	M 0.000073	M 0.000069	M 0.000069	M 0.000037
	Percentage decrease on basis of 0.000073 as standard....		5 per cent	5 per cent	50 per cent
When dye solution is stirred and changed every 5 sec.	Amount of dye in sap.....	M 0.00012			M 0.000056
	Percentage decrease on basis of 0.00012 as standard....				47 per cent

The third lot of cells was first exposed to the acetate buffer solution at pH 5.1 for 10 minutes, after which they were removed, wiped, washed for 5 seconds in phosphate buffer mixture at pH 6.6, again wiped, and placed in the 0.00035 M dye solution at pH 6.6 (phosphate buffer mixture). After 1 minute they were removed from the dye solution and the concentration of the dye in the sap was determined colorimetrically, and was found to be 0.000037 M.

The fourth lot of cells was taken directly from the tap water (at pH 7.7) and placed in the same dye solution as the third lot of cells. At the end of 1 minute the concentration of the dye in the sap was found to be 0.000073 M.

Cells thus treated did not live so well as the control cells when replaced in tap water so that in all probability they were more or less injured, but during the experiment the actual appearance of the cells, in respect to chlorophyll arrangement and turgidity, seemed about the same as that of control cells except that the sap appeared slightly murky. Cells kept continuously in the acetate buffer solution began to die in about 3 hours, so that after an exposure of 10 minutes there may have been a very slight injury.

Thus these experiments show that the decrease in the pH value of the sap brought about by acetic acid may be associated with a decrease^{8,9} in the rate of penetration of dye amounting to about 50 per cent, as shown in Table I.

This fact is of particular interest in connection with the theory^{1,4} that the dye is chiefly in the form of free base (for convenience called DB), at high pH values, and that this alone can penetrate the proto-

⁸ This decrease in the rate of penetration of dye is not due to the lowering of pH value of the external solution immediately surrounding the cell wall as a result of diffusion of acetic acid from the vacuole, because when the experiment is repeated by stirring the external solution, the relative amount of decrease in the rate is about the same as when the external solution is not stirred, as shown in Table I. Furthermore, this decrease is not caused by the adhering of acetic acid to the surface of cell wall in such a manner that it cannot be removed by washing and wiping before the cells are placed in the dye solution, because when the cells are placed in the dye solution, after they have been dipped in the acetate buffer solution only for 5 seconds or for 1 minute instead of 10 minutes, during which exposure the pH value of the sap remains normal, there is no decrease in the rate of penetration of dye, as shown in Table I.

⁹ This result is contrary to the result obtained with Cambridge *Nitella* previously described (Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 566, Foot-note 11) where an increase in the rate of penetration took place, but the extent of this increase was not so great (about 25 per cent). Since this work on Cambridge *Nitella* was done in midwinter, the experiments were repeated with the cells obtained in the summer, and it was found that in the majority of cases a decrease took place (about 25 per cent), which is less than the decrease in the case of New York *Nitella*. Such a difference in the behavior of cells may be due to the difference in the condition of the protoplasm.

plasm and enter the vacuole, and that the extent of accumulation of the total dye is dependent on the extent of change of this form, DB, on entering the vacuole into another form, DS, which cannot pass through the protoplasm. In that case we might expect the rate of penetration into the vacuole to be increased when the pH value of the sap is decreased, since with this decrease in the pH value of the sap the ratio of DB/DS decreases in the sap so that as DB enters the vacuole more of it will change to DS, thus causing more DB to enter. But since the experimental results give evidence to the contrary it is evident that the factor which controls the rate of penetration of dye into the vacuole cannot be wholly dependent on the condition of the sap. Under the present experimental conditions the rate of penetration of the dye must be controlled primarily by the effect of the acetate buffer on some other part of the cell. A series of experiments was therefore undertaken to determine the cause of this decrease in the rate of penetration of dye into the vacuole.

IV.

Can the Decrease in the Rate of Penetration of the Dye be Produced without Change in the pH Value of the Sap?

If the theory⁴ outlined in Sections I and III were correct we might assume that the decrease in the rate of penetration of dye associated with a decrease in the pH value is due to a change either at the surface or inside the protoplasm caused by the acetate buffer mixture. In that case we might very well expect a decrease in the rate of penetration when the cells are exposed¹⁰ to the solution only long enough for the protoplasmic surface or the interior of the protoplasm to be affected before a change in the pH value of the sap occurs. Unfortunately it is not possible to use the acetate buffer solution for this purpose since the pH value of the sap changes after a very few

¹⁰ The detailed description of the method of experimentation will be omitted hereafter since it is given in Section III. It may be repeated here that in all cases the cells were washed for about 5 seconds in a buffer solution at the same pH value as that of the dye solution before they were placed in the dye solution and the cells were invariably wiped before they were placed in any solution. Cells were exposed for 1 minute in the solution of dye, 0.00035 M made up with phosphate buffer mixture at pH 6.6 unless otherwise stated.

minutes exposure of the cells to the solution, even at the highest possible pH value (pH 5.4, M/150 acetate buffer mixture). For this reason it was necessary to expose the cells to a phosphate buffer solution at pH 5.4 for 10 minutes, in which the pH value of the sap remained unchanged, and to compare the rate of penetration of dye in the case of cells thus treated with the rate in the case of cells previously exposed for the same length of time in an acetate buffer solution at pH 5.4 where the pH value of the sap decreased from 5.5 (normal) to 5.0. As shown in Table II, there is about the same

TABLE II.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when the cells are placed in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after a previous exposure of the cells for 10 minutes either to the M/150 acetate buffer solution (when the pH of the sap is decreased) or to the M/150 phosphate buffer solution (when the pH value of the sap is not decreased).

External solutions.	In tap water at pH 7.7.	In acetate buffer solution at pH 5.4.	In phosphate buffer solution at pH 5.4.
	M	M	M
Amount of dye in sap.....	0.000073	0.000039	0.000041
Percentage decrease on basis of 0.000073 as standard.....		47 per cent	44 per cent

amount of decrease¹¹ in the rate of penetration whether the pH value of the sap is lowered or remains normal. The mortality of the cells thus treated is lower than that of the cells exposed to the acetate buffer mixture.

¹¹ Since there is about the same amount of decrease in the rate of penetration of dye whether the pH value of the sap is decreased or not, such a decrease cannot be due primarily to the decreasing of the pH value of the film⁴ of liquid between the protoplasmic surface and the cell wall as result of diffusion of acetic acid from the vacuole into the film. This film is the only part of the external system which affects penetration since it alone determines the number of dye molecules striking the protoplasmic surface. The condition of the external solution may be regarded as of importance only in so far as it affects this film.

V.

Is the Decrease in the Rate of Penetration of the Dye Due to the Effect of H Ions on the Surface or to Their Penetration (as Ions) or to the Entrance of Acids in Undissociated Form?

The decrease in the rate just described was about the same whether the pH value of the sap was lowered or not, and this suggests that the decrease in the rate might be due to the direct action of H ions on the surface or their penetration as ions when the pH value of the external solution changed from pH 7.7 (tap water) to pH 5.4 (buffer solutions). If this assumption were correct we might expect the rate of penetration to be about the same whether the cells were previously exposed to tap water, to phosphate, or to borate buffer solutions at pH 7.7 providing equal numbers of hydrogen or hydroxyl ions enter in each case.

In order to test this the rates of penetration of dye were compared among the three groups of cells previously placed¹⁰ for 10 minutes (1) in tap water (control), (2) in phosphate buffer solution, and (3) in borate buffer solution, all at pH 7.7, and it was found (as shown in Table III) that with phosphate buffer solution there was about 30 per cent less dye in the vacuole than in the case of the control, and with borate buffer about 13 per cent less dye (which may not be significant since the probable error of the mean is rather high).

The experiments were extended to higher pH values, pH 8.1 and 7.3, and it was found, as shown in Table III, that the rate of penetration of dye is again lower in the case of cells previously exposed¹⁰ to the phosphate buffer solution than that in the case of cells exposed to the borate solution. Such a difference in behavior between the borate and the phosphate buffer mixtures cannot be due to the effect of H or OH ions as such on the cell, since the pH value is the same in both these solutions.

Other experiments are therefore needed to determine just what causes this difference.

This difference between the phosphates and the borates, as affecting the rate of penetration of dye, is not due to the difference in the effect¹²

¹² It is not possible, unfortunately, to determine if there is an effect of acetate buffer mixture on the dye, since it is impossible to determine the penetration of dye at a pH value lower than pH 6.2 in the case of *Nitella*.

of these buffer mixtures directly on the dye, as is proved by the fact that when the cells are transferred¹⁰ directly from the tap water to

TABLE III.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when cells were previously exposed to M/150 borate and phosphate buffer solutions at different pH values for 10 minutes after which they were placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture).

External solutions.	In tap water at pH 7.7.	In phosphate buffer solution at pH 7.7.	In borate buffer solution at pH 7.7.	In phosphate buffer solution at pH 8.1.	In borate buffer solution at pH 8.1.
	M	M	M	M	M
Amount of dye in sap.....	0.000079	0.000055	0.000069	0.000059	0.000079
Percentage decrease on basis of 0.000079 as standard.....		30 per cent	13 per cent	26 per cent	0 per cent

External solutions	In phosphate buffer solution at pH 7.3.	In borate buffer solution at pH 7.3.	In phosphate buffer solution at pH 6.6.	In borate buffer solution at pH 8.7.	
	M	M	M	M	
Amount of dye in sap.....	0.000052	0.000069	0.000048	0.000079	
Percentage decrease on basis of 0.000079 as standard.....	35 per cent	13 per cent	39 per cent	0 per cent	

TABLE IV.

Comparison of the amount of brilliant cresyl blue in the sap after 1 minute in 0.00017 M dye solutions at pH 7.7 made up with different buffer mixtures (M/150).

External dye solutions.	Borate buffer mixture.	Phosphate (ordinary) $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$	Phosphate (lacking K) $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$
	M	M	M
Amount of dye in sap.....	0.00041	0.00035	0.00040

the dye solution at pH 7.7 made up (1) with borate buffer mixture and (2) with phosphate buffer mixture, the rate of penetration of the dye is the same, as shown in Table IV.

This difference furthermore is not due to a specific action of the K in the phosphate buffer mixture since the experiments were repeated¹⁰ with the solution made up with Na_2HPO_4 containing NaH_2PO_4 instead of KH_2PO_4 , at pH 5.4 and 7.7 and the same result was obtained, as shown in Table V.

TABLE V.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella* after 1 minute exposure to 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) following a 10 minute exposure to the M/150 phosphate buffer mixtures consisting of Na_2HPO_4 and either KH_2PO_4 or NaH_2PO_4 .

External solutions.	In tap water at pH 7.7.	In Na_2HPO_4 + KH_2PO_4 at pH 7.7.	In Na_2HPO_4 + NaH_2PO_4 at pH 7.7.	In Na_2HPO_4 + KH_2PO_4 at pH 5.4.	In Na_2HPO_4 + NaH_2PO_4 at pH 5.4.
	M	M	M	M	M
Amount of dye in sap.....	0.000084	0.000055	0.000059	0.000048	0.000048
Percentage decrease with 0.000084 as standard.....		33 per cent	29 per cent	42 per cent	42 per cent

TABLE VI.

Comparison of amount of brilliant cresyl blue in the vacuole when cells of *Nitella* (autumn) are placed in 0.00004 M dye solution (stirred) at pH 7.7 (M/150 borate buffer) for $\frac{1}{2}$ minute, after they have been exposed for 10 minutes to various solutions.

External solutions.	Tap water pH 7.7.	Boric acid pH 4.8.	Hydrochloric acid pH 4.8.	Phosphoric acid pH 4.8.	Phosphate buffer pH 5.4.
	M	M	M	M	M
Dye in sap.....	0.000072	0.000076	0.000069	0.000058	0.000042
Percentage decrease on basis of 0.000072 as standard.....		5 per cent increase (?)	5 per cent	20 per cent	42 per cent

The inhibiting effect of phosphate buffer mixtures is greater the lower the pH value, as shown in Table III. It may be that this is due to the greater amount of phosphoric acid present in the buffer mixture, if we assume that as a weak acid it penetrates the protoplasm as undissociated molecules and dissociates after entering and

lowers the pH value of the protoplasm, so that when cells are subsequently placed in a dye solution there will be less DB (since DB changes to DS more at a low pH value) in the protoplasm than in the case of the control cells which are transferred directly from tap water to the dye solution. The rate of penetration of DB from the protoplasm to the vacuole will therefore be less than in the case of the control cells.

This assumption¹² is partly supported by the following result. When the cells¹² are exposed for 10 minutes to three separate solutions, (1) hydrochloric acid, (2) boric acid, (3) phosphoric acid, all at pH 4.8, and then placed in the dye solution¹² (borate) for $\frac{1}{2}$ minute, the rate of penetration of dye (as compared with that of the control cells, which are transferred directly from tap water to the same dye solution) in the case of hydrochloric acid and boric acid is about the same as that of the control. This indicates that H ions do not affect¹² the cell and that if boric acid enters the cell as undissociated molecules it does not afterward dissociate sufficiently to lower the pH value to any appreciable degree. Phosphoric acid behaves differently in that the rate of penetration of the dye in the case of the cells exposed previously to this acid is found to be about 20 per cent lower than that of the control, which indicates that phosphoric acid enters the

¹² There are several other possible explanations, for example:

(1) On the basis that phosphoric acid enters more rapidly than boric. We are unable to prove this experimentally, for which reason the explanation described in the text is used instead.

(2) On the basis that a weak acid enters the protoplasm as undissociated molecule and by dissociating lowers the pH value of the protoplasm and that when such cells are removed from the buffer solution to the dye solution, the weak acid diffuses out from the protoplasm into the film of liquid between the protoplasmic surface and the cell wall, and lowers the pH value of the film thereby decreasing the ratio of DB/DS in the film. This will explain the difference between boric acid and phosphoric acid, in that boric acid does not change the pH value of the film since it is too weak an acid, while phosphoric acid is sufficiently strong to bring about this change. But this assumption is not so satisfactory as the one described in the text when we consider the fact that there is an inhibiting effect on the rate of penetration of dye even with cells previously exposed to a phosphate buffer solution at pH 8.1 and then placed in dye solution at much lower pH value (pH 6.6). In such a dye solution one would expect further entrance of phosphoric acid into the cell, rather than exit of the acid from the protoplasm to the exterior of the cell. These cells were collected in autumn.

The dye solution was stirred. At a lower pH value both phosphoric acid and hydrochloric acid have an inhibiting effect which is greater in the case of the former.

protoplasm and then dissociates sufficiently to lower the pH value or else that it has a specific effect on the surface (Table VI).

The cause of the decrease brought about by the phosphate buffer mixture may be threefold, (1) due to undissociated phosphoric acid, (2) due to the Na and K salts present in the buffer mixture, and (3) due possibly to certain anions.

It may be of interest to add here the following. When cells (collected in Cambridge) are exposed for 10 minutes to solutions at different concentrations (0.05 M to 0.006 M) of NaCl, LiCl, KCl, Na₂SO₄, and NaNO₃ made up in distilled water, after which they are washed in distilled water for 5 seconds, wiped, and are placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) for 1 minute, the rate of penetration is considerably decreased as compared with the control. If cells are placed directly for 1 minute (without such treatment) in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) containing any one of these salts, the rate is found to be slightly higher than in the case of cells placed in dye solution containing no salt.

Solutions of MgCl₂, MgSO₄, CaCl₂, LaCl₃, and LaNO₃ all behave alike, in that when cells are exposed to these solutions for 10 minutes and then transferred to the dye solution, the rate of penetration of dye is about the same as the control. When cells are placed without such treatment in dye solutions containing any one of these salts (LaCl₃ omitted), the rate is found to be somewhat higher than that of the control.

Thus there is evidence for the inhibiting effect¹⁴ of the salts with

¹⁴ The experiments described in the text (see Table III) show that the borate buffer mixtures have no inhibiting effect on the rate of penetration of dye. In view of the fact that the borate buffer mixtures at higher pH values contain a considerable amount of Na, there is an apparent discrepancy between the results obtained in this case and those in the case of NaCl solutions in which there is a considerable inhibiting effect due to the presence of Na (this discrepancy was mentioned in the writer's previous paper (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, No. 1)). This, however, may be due to the fact that in the case of cells previously exposed to the borate buffer mixtures the dye was made up with phosphate buffer mixtures which seem to diminish the inhibiting effect of Na, while in the case of cells previously exposed to NaCl solutions the dye used was made up with borate buffer mixture which does not seem to have this effect. The following experiments may make this clear. When cells previously exposed to (1) 0.01 M NaCl and (2) to 0.005 M sodium borate solutions for 10 minutes were washed for 5 seconds in distilled water, wiped, and placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture), there is a considerable inhibiting effect

monovalent base cations which is not easily reversible, since the effect may be brought about by placing the cells in 0.01 M NaCl solution for 5 minutes, but this effect does not disappear after the cells have been transferred to distilled water and left for over an hour. This effect, however, may be readily removed if cells are placed in a solution of salt with bivalent or trivalent cations, such as $MgCl_2$ and $LaCl_3$ at certain concentrations.

Further experiments are being carried out on this subject by the writer.

VI.

Experiments on Valonia.

The experiments have been repeated with *Valonia macrophysa* but the results described below are approximate and show only relative values, owing to the fact that the pH value of the sap cannot be accurately determined without special experiments and that the sea water not only shifts the dissociation constant considerably, but seems to change the nature of the dye, especially at lower pH values.

A. The Effect of Aqueous Ammonia (Free and Combined).—Since the method of determining the change in the pH value after placing cells in solutions has been described in detail in Section II, it will be omitted here. When cells of *Valonia* were placed for 1 hour in sea water containing 0.003 M NH_4Cl solution, the pH of the sap increased from 6 (normal) to 6.6 (determined colorimetrically by using brom-cresol purple). When such cells were replaced in sea water and left for 1 hour the pH value decreased from 6.6 to 6.1.

One group of cells was placed in sea water containing 0.00035 M dye, a second group in sea water containing 0.003 M NH_4Cl and

which is slightly greater with (1) than with (2). If such cells (1) and (2) are placed in 0.00014 M dye solution at pH 7.7 (phosphate buffer solution) they show no inhibiting effect at all.

At a higher concentration of NaCl (0.05 M) this inhibiting effect is not removed in 0.00014 M dye made up with phosphate buffer mixture at pH 7.7.

The inhibiting effect of previous treatment with the phosphate buffer mixture at pH 5.4 (Table II) is increased in 0.00014 M dye solution at pH 7.7 made up with borate buffer mixture.

0.00035 M dye. A third group of cells was first exposed for 1 hour to sea water containing 0.003 M NH_4Cl and then transferred to the dye solution used in the case of Group 1. After 1 hour there was a decided decrease in the rate of penetration of dye in the case of cells placed in the dye solution containing NH_4Cl (Group 2) and also in the case of cells previously exposed to NH_4Cl solution (Group 3), as compared with the control (Group 1). These results show that the presence of ammonia in the cell brings about a decrease in the rate of penetration of dye. Whether this decrease is entirely due to the increase in the pH value of the sap in the presence of ammonia or due partly to the former and partly to the presence of ammonia in the protoplasm (at the surface or the interior), it is not possible to determine. These results confirm those obtained with *Nitella*³ (see Section I).

B. Effect of Acetic Acid and HCl at pH 5.9.—Let us first see if the same results may be obtained as with *Nitella* when the pH value of the sap is decreased by entrance of acetic acid. When cells were placed in sea water containing acetic acid at pH 5.9. the pH value of the sap decreased in 1 hour from 5.5 (normal) to 4.8 (methyl¹⁵ red used as an indicator). The pH value of the sap thus decreased was found to be raised to the normal when such cells were placed in sea water for 20 minutes. When cells whose pH value had been thus decreased were placed for 20 minutes in sea water containing 0.00035 M dye, the amount of dye in the sap was less¹⁶ than in the sap of cells transferred directly from the sea water to the same dye solution (control). These experiments show that there is a decrease in the rate of penetration of dye when the pH value of the sap is decreased

¹⁵ Difference between the determination of the pH value of the sap made with brom-cresol purple and with methyl red lies in the fact that the effect of salt on the indicator is not corrected. The explanation of the use of the indicators is described in Section I.

¹⁶ Brooks exposed cells of *Valonia macrophysa* to sea water (1) containing NH_4Cl until the pH value of the sap increased, and (2) containing CO_2 until the pH value of the sap decreased, after which they were placed in sea water containing 2, 6, dibromophenol indophenol, and found that the rate of penetration of dye decreased with (1) and increased with (2). She interprets these results on the basis that the rate of penetration of dye is affected by the change in the pH value of the external solution surrounding the cell as a result of diffusion of (1) NH_4Cl and (2) CO_2 from the vacuoles. (See Foot-note 6.)

by entrance of acetic acid, which agrees with the results discussed in Section III on *Nitella*.

Let us now see if a decrease can be brought about without a change in the pH value of the sap. Cells were exposed for 1 hour to sea water containing HCl at pH 5.9, after which they were placed in 0.00035 M dye for 20 minutes. The rate of penetration in this case was found to be less than the control but the extent of decrease in the rate is not so great as it was in the case of cells exposed to acetic acid.

When cells are placed for 1 hour in sea water containing 0.0007 M dye at pH 5.9, (1) containing acetic acid and (2) containing HCl, the rate of penetration was found to be higher with acetic acid than with HCl.

C. Effect of Sea Water at pH 6.5 Containing either Acetic Acid or HCl (No Change in the pH Value of the Sap).—The question now arises as to what will happen if we put cells in sea water containing acetic acid at a pH value at which there is no decrease in the pH value of the sap. Cells were placed in sea water at pH 6.5 containing acetic acid for 1 hour after which they were transferred to sea water containing 0.00035 M dye for 20 minutes: the pH value of the sap remained normal. When the rate of penetration of dye in the case of the cells thus treated was compared with that of the control (cells directly removed from the sea water and placed in the same dye solution), it was found to be the same. In the case of the cells previously exposed to sea water containing HCl at pH 6.5 the rate of penetration of dye was also found to be the same.

Cells placed in 0.00017 M dye in sea water at pH 6.5 containing (1) acetic acid and (2) HCl, showed no difference in the rates.

Thus these experiments show that in the case of *Valonia* also the rate of penetration of dye may be retarded when (1) the pH value of the sap is decreased in presence of acetic acid, and (2) the pH value of the sap is increased in presence of NH_3 , when cells are exposed to these solutions before they are placed in the dye solutions.

SUMMARY.

When living cells of *Nitella* are exposed to an acetate buffer solution until the pH value of the sap is decreased and subsequently placed in a solution of brilliant cresyl blue, the rate of penetration of dye into the vacuole is found to decrease in the majority of cases,

and increase in other cases, as compared with the control cells which are transferred to the dye solution directly from tap water. This decrease in the rate is not due to the lowering of the pH value of the solution just outside the cell wall, as a result of diffusion of acetic acid from the cell when cells are removed from the buffer solution and placed in the dye solution, because the relative amount of decrease (as compared with the control) is the same whether the external solution is stirred or not.

Such a decrease in the rate may be brought about without a change in the pH value of the sap if the cells are placed in the dye solution after exposure to a phosphate buffer solution in which the pH value of the sap remains normal. The rate of penetration of dye is then found to decrease. The extent of this decrease is the greater the lower the pH value of the solution.

It is found that hydrochloric acid and boric acid have no effect while phosphoric acid has an inhibiting effect at pH 4.8 on stirring.

Experiments with neutral salt solutions indicate that a direct effect on the cell (decreasing penetration) is due to monovalent base cations, while there is no such effect directly on the dye.

It is assumed that the effect of the phosphate and acetate buffer solutions on the cell, decreasing the rate of penetration, is due (1) to the penetration of these acids into the protoplasm as undissociated molecules, which dissociate upon entrance and lower the pH value of the protoplasm or to their action on the surface of the protoplasm, (2) to the effect of base cations on the protoplasm (either at the surface or in the interior), and (3) possibly to the effect of certain anions. In this case the action of the buffer solution is not due to its hydrogen ions.

In the case of living cells of *Valonia* under the same experimental conditions as *Nitella* it is found that the rate of penetration of dye decreases when the pH value of the sap increases in presence of NH_3 , and also when the pH value of the sap is decreased in the presence of acetic acid. Such a decrease may be brought about even when the cells are previously exposed to sea water containing HCl , in which the pH value of the sap remains normal.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

SUSCEPTIBILITY OF RABBITS TO INFECTION BY THE INHALATION OF VIRULENT PNEUMOCOCCI.

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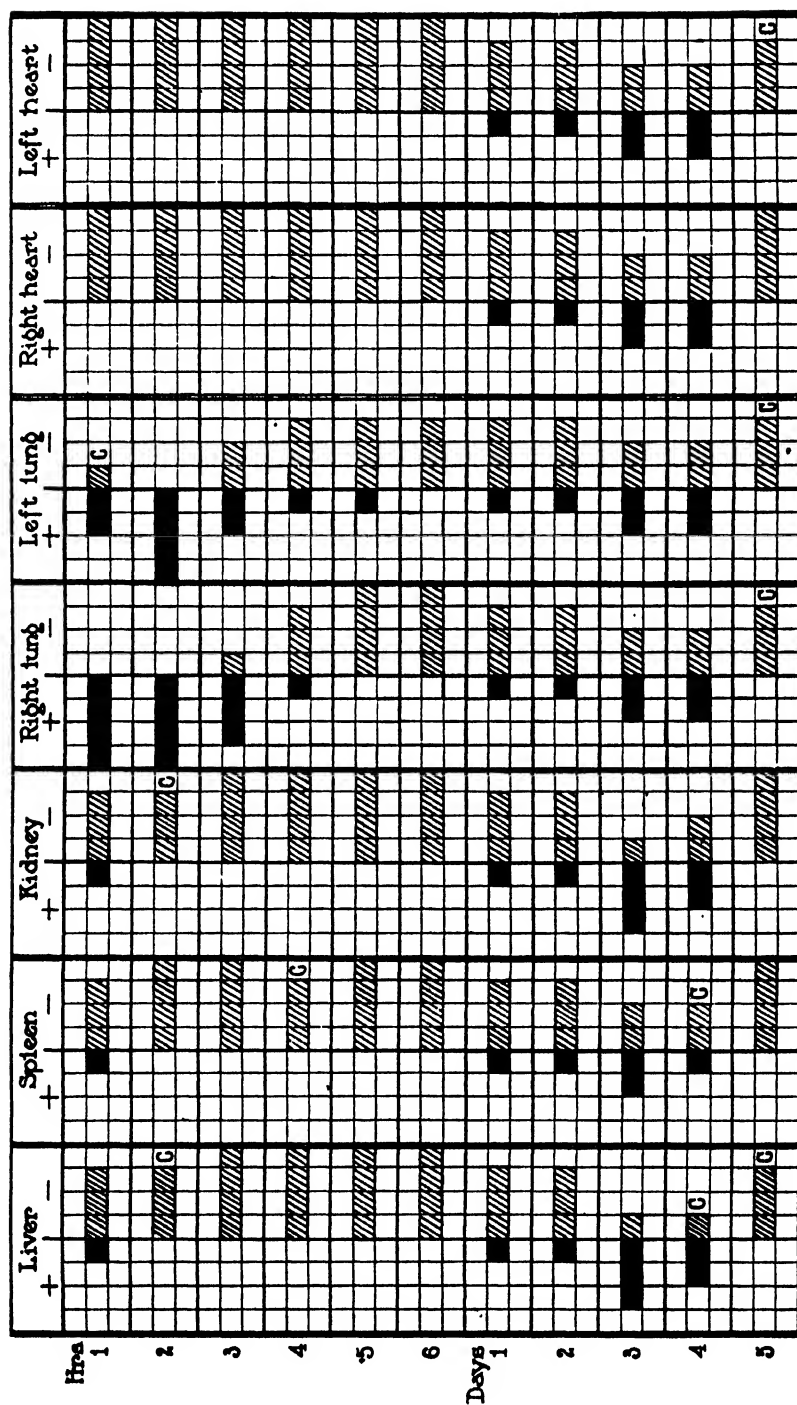
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In preceding papers (1, 2) it has been shown that following inhalation, virulent pneumococci generally disappear from the lungs of normal mice within a few hours, that infection rarely occurs, and that in only a few instances does death result from septicemia. It has further been shown that a high degree of active immunity may develop in mice following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the varying degrees of immunity developed by the repeated inhalations. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

In the present paper are reported (1) the distribution of pneumococci in the organs of rabbits following spraying with pneumococci, (2) the occurrence of positive blood cultures, (3) the mortality of the exposed rabbits, and (4) the pathology of the pulmonary lesions in the rabbits which died. The immunity following upon the inhalation of pneumococci will be dealt with in a subsequent paper.

Method.

The rabbits were placed in a large spray chamber similar to that already described (2) and exposed to a spray of virulent Type I pneumococci. 150 cc. of an 18 hour broth culture was used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained by bleeding the animal from the ear vein. At varying intervals after the spraying, blood cultures were taken from the ear vein. In the case of the animals which were killed and autopsied, broth cultures were made from both sides of the heart, and small pieces of the periphery of both lungs, and the kidney, liver, and spleen were placed directly in tubes of broth. Heart's blood cultures alone were made from the rabbits which died. All cultures were plated on blood agar for further identification. The animals killed with chloroform were immersed in a solution of lysol and opened with sterile instruments.



TEXT-FIG. 1. Distribution of pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile.

Distribution of Pneumococci in the Organs.

In Text-fig. 1 is shown the distribution of pneumococci in the organs of 44 sprayed rabbits which were killed at intervals after spraying. It will be seen from the figures for the 24 rabbits killed in from 1 to 6 hours that pneumococci were generally recovered from the periphery of the lungs for the first 3 hours after the spraying but that later they rapidly disappeared. In only 1 rabbit were pneumococci recovered from the liver, spleen, and kidney. The heart's blood cultures from this animal remained sterile. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, 6, or 30 per cent, showed a general pneumococcus septicemia. In 1 rabbit killed on the 3rd day, pneumococci were recovered only from the liver and kidney. This experiment shows how readily normal rabbits may become infected following inhalation of pneumococci. It further indicates that a transient pneumococcus septicemia may be present following spraying. In order to gain further knowledge of this point daily blood cultures were taken from the ear vein of a few rabbits.

Blood Cultures.

From Table I it is seen that following a first exposure positive blood cultures were obtained in 15 out of 44 rabbits. Of the 15 rabbits which developed a septicemia 5, or 33 per cent, continued to have a positive blood culture until death. The other 10 rabbits had merely a transient septicemia and survived. In some instances the septicemia could be detected during the first 24 hours but in others the organisms were first recovered as late as the 6th or 7th day.

Mortality of Rabbits Following Exposure to a Pneumococcus Spray.

Table II shows the number of normal rabbits which succumbed during the course of spraying with virulent pneumococci. From this table it is shown that out of 231 rabbits 80, or 38 per cent, died of a pneumococcus septicemia following their first exposure. Although some rabbits escaped infection at the first exposure, 7, or 6 per cent, died after the second spraying, and an occasional rabbit died after the third and fourth exposures. Of the 112 rabbits which died, the blood of 13, or 11 per cent, was sterile. These rabbits probably succumbed

TABLE I.

Days on Which Positive Blood Cultures Were Obtained Following a Single Exposure to a Pneumococcus Spray.

Rabbit No.	Days..1	2	3	4	5	6	7	8	9	Course
1	c	c	-	-	-	+				Survived
2	-	+	+	-	-					"
3	+	+	+	+						Died 6th day
4	+	-	-	-	-	-				Survived
5	-	+	c	c		+				Died 7th day
6	-	+	+	c	c					" " "
7	-	-	-	c	+		-			Survived
8	+		c			-				"
9	-	-	-	+						Died 6th day
10	-	c	+	+						" 5th "
11	+	c	-	-	-					Survived
12	+	+	-		-	-	-	-	-	"
13	+	-	-		-	-	+	c	-	"
14	-	-	-		-	-	+	-	-	"
15	-	-	-		c	-	+	-	-	"

Blood cultures in 29 rabbits were negative.

A cross indicates a positive culture of pneumococcus, a dash a sterile culture, and "c" a contaminated culture.

TABLE II.

Fate of Rabbits after One to Ten Exposures to Inhalations of Pneumococci.*

No. of times exposed to virulent pneumococci	No. of rabbits exposed	No. dying	Pneumococcus recovered from heart's blood	Pneumococcus not recovered from heart's blood
1	231	86	80	6
2	111	9	8	1
3	98	11	9	2
4	71	4	2	2
5	50	1	-	1
6	39	1	-	1
7	34	-	-	-
8	31	-	-	-
9	31	-	-	-
10	27	-	-	-

* The discrepancy between the total numbers and the sum of the numbers in the individual groups is due to the fact that certain of the animals that survived were used for intraperitoneal tests or for other experiments.

to suffocation from being enclosed in the spray boxes too long. This technical error was corrected in later experiments. The table also brings out the fact that the greatest number of deaths occurred after the first exposure. After each successive spraying the mortality tended to lessen until after the fourth exposure no animals succumbed. In other words, those rabbits which were most susceptible to pneumococcus died early. As will be shown in a subsequent paper, the surviving rabbits were those which not only had the greatest natural resistance but also had acquired a certain degree of immunity as a result of repeated exposures to pneumococcus.

The interval elapsing between the time of spraying and death from pneumococcus septicemia is shown in Table III. From this it is seen that the greatest number of animals died within the first 7 days fol-

TABLE III.

Number of Days Elapsing between Spraying and Death of Rabbits from Pneumococcus Septicemia.

	Days..1	2	3	4	5	6	7	8	9	10
Spray I.....	3	12	16	8	14	12	8	5	1	1
" II.....	—	2	—	3	1	—	—	—	1	1
" III.....	2	—	2	—	—	1	2	2	—	—
" IV.....	—	—	1	—	—	1	—	—	—	—

lowing the initial exposure, although an occasional rabbit survived longer. The time of death of the rabbits which had survived the first exposure is much more diverse, and these rabbits have a tendency to die after a longer interval.

Pathology.

In no instance was there any gross evidence of pneumonia, although serous and serofibrinous pleurisy and serofibrinous pericarditis were common. A histological examination was made of the lungs of 77 rabbits which died of pneumococcus septicemia following spraying. The lungs appeared normal in 20. Congestion and pleurisy were noted in 7 instances, edema in 25, congestion in 38, and hemorrhagic extravasation into the alveoli in 19. In only 1 instance was there any histolog-

ical evidence of an attempt at localization of the infection. The lungs of this rabbit which died on the 4th day following the first exposure showed an interstitial inflammation (3).

DISCUSSION.

From the foregoing experiments it appears that rabbits are even more susceptible than mice to infection with pneumococci following inhalation. For a period of 3 hours after spraying the organisms may be demonstrated at the periphery of the lungs. It is unfortunate that their subsequent course deeper into the lungs cannot be followed. But the great number of other organisms which normally inhabit the bronchi of rabbits, as Jones (4) has shown, renders cultural studies of the lungs difficult. Histological detection of pneumococci in the tissues is also unreliable when the organisms are present only in small numbers.

In all probability the majority of the organisms are destroyed either when they first localize or deeper in the lung tissue by the endothelial leucocytes or by polymorphonuclear leucocytes. In any case a few organisms occasionally filter through into the blood stream. Gaskell (5) believes that invasion of the blood stream by pneumococci probably always takes place in the early stages of an air-borne infection of the lung. The actual invasion of the blood stream must be much more common than is supposed. Not only are there probably few organisms free at any one time in the circulating blood but these few bacteria may even be within leucocytes and not in reality multiplying in the blood.

The occasional organisms which reach the blood are probably rapidly filtered out of the blood stream by the organs and locally destroyed. It is evident, however, that following the apparent disappearance of the pneumococci from the periphery of the lungs, they again appear, this time in the blood, and as a result may be recovered from all the organs. The point of this reinvasion is as yet uncertain.

Following the initial spraying with pneumococci the majority of susceptible rabbits die of an overwhelming septicemia without any attempt on the part of the body to localize the infection.

CONCLUSIONS.

1. Rabbits are very susceptible to infection by inhalation of Type I pneumococci.

2. When rabbits are exposed to a pneumococcus spray the bacteria readily penetrate into the lower respiratory tract. The pneumococci which reach the periphery of the lungs as a result of this procedure usually disappear within a few hours but a generalized and fatal septicemia frequently later appears. Pneumococci may then be recovered from the periphery of the kidney, liver, and spleen. In the animals which die pleurisy and pericarditis are common but pneumonia does not occur.

3. Rabbits may recover from pneumococcus septicemia.

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IMMUNOLOGICAL RELATIONSHIPS OF ENCAPSULATED AND CAPSULE-FREE STRAINS OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

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In a preceding paper (1) evidence was presented that bacilli of the Friedländer group are separable into sharply defined and specific types. Of 30 strains employed in the study, three specific types and a heterogeneous group were demonstrated by agglutination, agglutinin adsorption, protection, thread and precipitin reactions. These types have been designated Type A, Type B, and Type C, while the remaining unclassified strains were placed in a tentative group, Group X. In the light of the present studies, the difficulties encountered in previous attempts to interpret the immunological reactions of Friedländer's bacillus appear to be due in large measure to the failure to distinguish in the cell the type-specific from the species-specific antigen; and the failure to recognize in immune serum the two distinct antibodies provoked by the respective antigens. The application of this concept to the Friedländer group was suggested by studies carried out in this laboratory on the immunological relationships of the cell constituents of *Pneumococcus* (2-7). Briefly, this concept involves two separable and distinct antigens—the one a soluble specific substance (now identified as a carbohydrate) which endows the organism with type specificity; the other, a protein substance which exhibits only the common and undifferentiated characters of the species.

Since the relation of the capsular material of Friedländer's bacillus to type specificity has been demonstrated (1, 8, 9), it seemed of importance to determine the immunological relationships of encapsulated and capsule-free strains of this organism. For this purpose, S and R varieties of the bacterial cell were obtained as will be de-

scribed later. It was observed that while S cells were encapsulated, the R forms no longer possessed capsules.

The nomenclature of S and R was first used by Arkwright (10) to describe biological variations in single strains of bacteria. His observations included the occurrence of two forms of colonies in old cultures of bacilli of the intestinal group. The S organisms grew in colonies with a smooth surface while the R strains presented colonies with a rough and irregular surface. The terms S and R have been universally adopted to describe similar changes in other species of bacteria. Observations on encapsulated organisms, however, fall mainly within the *Pneumococcus* group. With these organisms, it has been shown that accompanying the change from S to R, there is a loss of agglutinability by specific sera, loss of capsule, and attenuation of virulence (10-19).

Cultures of Friedländer's bacillus, also, have been separated by earlier workers (20-24) into S and R components, although such a designation was not in use at the time. The method of decapsulation as devised by Porges (25) must also be considered of the same order. More recently, Friel (13) and Hadley (26) have reported the occurrence of the two varieties in individual cultures of Friedländer's bacillus and have attributed to them the accepted characteristics of S and R forms.

In this study, the criteria for the conversion of S organisms to R forms have been: (1) loss of capsule and mucoid characteristics, (2) loss of agglutinability in type-specific sera, (3) attenuation of virulence, and (4) the development of colonies which present under the microscope a rough and irregular surface. In the case of R cells derived from Type C, loss of virulence was not an accompanying change since none of the Type C strains were virulent for mice.

The present report deals with the immunological reactions of encapsulated and capsule-free strains of Friedländer's bacilli and anti-S and anti-R sera, and the serological behavior of encapsulated bacilli after removal of capsules by the chemical method of Porges.

Methods.

Loss of Capsule Formation Induced by Growth in Vitro.—The non-encapsulated or "R" strains employed in this study were derived from pure S strains. An S strain representative of each of the three fixed types and one strain chosen from Group X were transplanted daily in broth to which had been added 10 per cent of homologous immune serum. Plates, streaked at the time of each transplant, were examined microscopically after 18-24 hours incubation, as recommended by Reimann (18). Within 6-10 transplants, plate cultures were obtained in which a

number of R colonies were observed. Single, typical R colonies were then transplanted into plain broth and the resultant R culture showed loss of capsule and attenuation of virulence. In each instance, the R culture failed to kill white mice, in doses of 0.5 cc., while the parent S strain from which the R had been derived regularly killed within 48 hours at a dilution of one ten-millionth cc.

Destruction of Capsule by Chemical Means.—The method devised by Porges was used. S strains were grown on agar slants and the organisms were washed off and suspended in salt solution. The suspensions were made acid with one-fourth volume N/4 HCl and heated at 80°C. for 15 minutes. The suspensions were then cooled immediately under tap water and neutralized with an equivalent quantity of N/4 NaOH. The exposure to heat was varied from 60°C. to 100°C. and from 10 minutes to 30 minutes without appreciable differences. No spontaneous clumping of the treated organisms was experienced when freshly prepared suspensions were used. After standing in the ice chest several days, however, preparations from two different strains became granular.

The method of immunization and the reaction of agglutination, precipitin and protection tests were conducted in the manner described in the preceding paper (1).

EXPERIMENTAL.

I. Immunological Reactions of "S" (Encapsulated) Strains in Immune Sera.

(a) *Anti-S Sera.*—It has already been shown in the preceding paper (1) that antisera prepared by immunization with encapsulated strains possess type-specific immune bodies, which agglutinate the encapsulated organisms and precipitate the soluble specific substance derived from them. In addition, such sera confer specific protection upon white mice against infection with strains of homologous types.

(b) *Anti-R Sera.*—Since the anti-R sera were prepared against strains of organisms which were capsule-free and which had lost the function of elaborating the type-specific soluble substance, it was to be anticipated that these sera would be lacking in type-specific antibodies. Definite proof of this, however, was obtained by determining the agglutination of S strains in anti-R sera. For purposes of comparison, the specific reactions of these same strains in anti-S sera of the homologous type were included. The results presented in Table I show conclusively that encapsulated strains are not agglutinated by sera prepared by immunization with the capsule-free (R) variants. This is true even though the organism employed for

immunization was derived from the S strain later used for agglutination.

TABLE I.

Agglutination of Friedländer S Strains by Anti-R Sera.

Antigen encapsulated strain	Anti-R sera						Anti-S sera			
	Type A		Type B		Group X		A	B	C	X
	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A.....	-	-	-	-	-	-	++++	-	-	-
" B.....	-	-	-	-	-	-	-	++++	-	-
" C.....	-	-	-	-	-	-	-	-	++++	-
Group X.....	-	-	-	-	-	-	-	-	-	++++

* The figures in this row represent ultimate dilution of serum.

++++ indicates compact disk agglutination with clear supernatant; -, no reaction.

TABLE II.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-R Sera.

Antiserum	Soluble specific substance of Friedländer's bacillus											
	Type A						Type B					
	*2	20	50	100	250	500	2	20	50	100	200	500
Type A (R).....	-	-	-	-	-	-	-	-	-	-	-	-
" B ".....	-	-	-	-	-	-	-	-	-	-	-	-
Group X ".....	-	-	-	-	-	-	-	-	-	-	-	-
Type A (S).....	-	+	++	++	+	-	-	-	-	-	-	-
" B ".....	-	-	-	-	-	-	++	+++	++++	++++	++++	++
Normal.....	-	-	-	-	-	-	-	-	-	-	-	-

* The dilutions are expressed in thousands.

++++ indicates heavy, compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity.

Further evidence of the lack of type-specific antibodies in anti-R sera was sought by the precipitin reaction. Solutions of the isolated soluble specific substances of Types A and B were tested against three anti-R sera. As demonstrated in Table II, these sera contained no pre-

cipitins for the type-specific substances just as in the preceding experiment they were shown to possess no agglutinins for the encapsulated cells from which these substances were derived.

It has been shown in the previous communication that anti-S sera contain antibodies which afford specific protection in mice against

TABLE III.

Protection Offered by Anti-R Sera against Infection by Type A, Friedländer's Bacillus.

Type A encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 16 hrs.	.2	D. 20 hrs.	
.0001	.2	" 19 "	.2	" 20 "	
.00001	.2	" 39 "	.2	" 22 "	D. 24 hrs.
.000001	.2	S.	.2	" 65 "	" 39 "
.0000001					" 65 "

S. indicates survival; D., death, the numerals representing the number of hours before death occurred.

TABLE IV.

Protection Offered by Anti-R Sera against Infection by Type B, Friedländer's Bacillus.

Type B encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 15 hrs.	.2	D. 15 hrs.	
.0001	.2	" 15 "	.2	" 15 "	
.00001	.2	" 39 "	.2	" 24 "	D. 16 hrs.
.000001	.2	" 22 "	.2	" 65 "	" 39 "
.0000001					" 39 "

infection with virulent encapsulated bacilli of homologous types. Accordingly, an analysis of anti-R sera was made to determine the presence of protective properties. The results of these determinations are given in Tables III and IV. An anti-R serum prepared by immunization with a degraded Type A organism affords no measura-

ble protection against infection with an encapsulated (S) strain of Type A or Type B. Similarly, an anti-R serum prepared by immunization with a capsule-free organism derived from Type B offers no protection against infection with virulent strains of either of these specific types.

In summary, then, the foregoing experiments show that antisera prepared by the immunization of rabbits with degraded, capsule-free strains of Friedländer's bacillus (R forms) are devoid of specific agglutinins, precipitins, and protective antibodies for the virulent, type-specific, encapsulated bacilli. Anti-R sera, therefore, exhibit none of the type-specific reactions which characterize anti-S sera.

II. Immunological Reactions of "R" (Non-Encapsulated) Strains in Immune Sera.

(a) *Anti-S Sera.*—The anti-S sera used for the determination of specific types (1) were prepared in such manner as to avoid or at least minimize the concurrent presence of the common specific antibody. As was pointed out in the preceding paper, this may be accomplished most successfully by using young cultures of encapsulated organisms and by avoiding prolonged immunization. It was considered of interest to determine the agglutinative action of the type-specific sera against R strains of different origin. The results of these tests are given in Table V. It is seen that an immune serum prepared against an encapsulated strain of Type A contains only a small amount of the species antibody. In addition, this antibody is shown to be equally operative against four R strains, each of which in turn had been derived from an S organism of a serologically different type. The three anti-S sera of Type B, Type C, and Group X contained only minute and, for practical purposes, negligible traces of the R or common species antibody. Thus, further confirmation is advanced for the concept that the R organisms, devoid of capsules, are no longer type-specific.

(b) *Anti-R Sera.*—Evidence has been presented that capsule-free strains of Friedländer's bacillus are lacking in the ability to engender type-specific antibodies. However, they stimulate the formation of antibodies which react not only with the particular strain used for immunization, but with all other R forms regardless of the type from

which they were derived. This fact is revealed in the cross-reactions of agglutination presented in Table VI in which all R strains are shown to be reciprocally agglutinated by each of the different anti-R sera.

The agglutination of the R strains in anti-R serum is characteristically different from the agglutination of S strains in anti-S serum of the homologous type. It will be recalled that with S strains, the reaction results in the formation of a compact, firm disk of agglutinated bacteria and occurs almost immediately in the more concentrated dilutions of the serum. The R organism, on the other hand, agglutinates very slowly, and in the higher concentrations of serum forms a fluffy precipitate; while in greater dilutions of the serum a fine, granular agglutination occurs which is difficult to read without a

TABLE V.

Agglutination of Friedländer R Strains by Anti-S Sera.

Antigen capsule-free strain	Anti-S sera											
	Type A			Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A.....	++	+	+	±	-	-	-	-	-	-	-	-
" B.....	++	+	+	±	-	-	-	-	-	-	-	-
" C.....	+	+	+	±	-	-	+	-	-	+	-	-
Group X.....	++	+	+	±	-	-	+	-	-	+	-	-

lens. The agglutinins in anti-R sera, which react with capsule-free strains, bear a marked similarity to the "fine flaking" agglutinins for the H form of *proteus* and typhoid bacilli described by Felix (27) and to the somatic agglutinins for the non-motile forms of hog-cholera bacillus reported by Orcutt (28). Furthermore, the agglutinin titre of anti-S sera is low (1:40-1:80) while the agglutinin titre of anti-R sera is high (1:2500).

Corroborative evidence of the serological identity of R strains was gained by the agglutinin adsorption test. Each anti-R serum was adsorbed with heat-killed suspensions of each R strain until all the agglutinins were removed for the adsorbing organisms. The adsorbed sera were then tested for the presence of agglutinins for other R strains. Repeated tests yielded constant results. To avoid un-

TABLE VI.
Cross-Agglutinations of R Strains of Friedländer's Bacillus by Anti-R Sera.

Anti-R sera	Antigen derived from	Final dilution of serum										Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Type A	Type A	++	++	++	++	++	++	++	++	++	+	
	" B	++	++	++	++	++	+	+	±	-	-	
	" C	++	++	++	++	++	++	++	+	-	-	
	Group X	++	++	++	++	++	++	+	+	-	-	
" B	Type A	++	++	++	++	++	++	++	+	+	-	
	" B	++	++	++	++	++	++	++	++	++	+	
	" C	++	++	++	++	++	++	++	++	++	-	
	Group X	++	++	++	++	++	++	++	++	++	+	
Group X	Type A	++	++	++	++	++	++	++	++	+	-	
	" B	++	++	++	++	++	++	++	+	-	-	
	" C	++	++	++	++	++	++	++	++	+	-	
	Group X	++	++	++	++	++	++	++	++	++	+	

+++ indicates complete agglutination with flocculent sediment and clear supernatant; ++, almost complete, supernatant clouded; +, marked agglutination; +, slight agglutination; -, no agglutination.

necessary repetition, a single typical protocol is given in Table VII. It is evident that the adsorption of an anti-R serum with any R strain removes from the serum agglutinins for the homologous organism as well as for heterologous strains. It is apparent, therefore, that immunologically all R cells are identical, as tested by the reactions of agglutination and agglutinin adsorption.

TABLE VII.

Agglutinin Adsorption.

Results of Agglutination with Anti-R Serum (Type B) after Adsorption by R Strains Derived from Homologous and Heterologous Types of Encapsulated Organisms.

Antigen capsule-free strain from	Anti-R serum (Type B) after adsorption with R strains derived from															
	Type A				Type B				Type C				Group X			
	*20	50	100	500	20	50	100	500	20	50	100	500	20	50	100	500
Type A.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
“ B.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
“ C.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Group X.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Figures represent ultimate dilution of serum.

III. Immunological Reactions of “S” (Encapsulated) Strains after Decapsulation by Porges’ Method.

Appreciating the difficulties in the agglutination of Friedländer’s bacillus, Porges devised a method of removing the capsule by weak acid in order to render strains more antigenic and better agglutinable. To test the effect of decapsulation on the serological behavior, suspensions of encapsulated bacilli representative of each of the specific types were subjected to the Porges technique. The cells were then tested for agglutinability in sera prepared by immunization with encapsulated and capsule-free organisms, respectively. For purposes of comparison, a strain of *Encapsulatus granulomatis* was included in the experiment.

(a) *Anti-S Sera*.—The results obtained with anti-S sera are summarized in Table VIII. It is evident that encapsulated organisms

which are heated in the presence of acid are not agglutinated appreciably in purely anti-S sera of types which are serologically different from that yielding the decapsulated cells. Agglutination in such sera depends upon the presence of species antibody. In anti-sera of the parent strains, however, there is a definite precipitin reaction which is referable to the presence of unhydrolyzed soluble specific substance. In this connection it will be recalled (1, 9) that the capsular material of Friedländer's bacillus is precipitated by anti-S serum of the homologous type. Since the method of Porges strips the bacillus of the capsule with apparently only partial hydrolysis, it is not surprising that precipitation of the soluble specific substance is observed.

TABLE VIII.

Agglutination by Anti-S Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Antigen derived from	Anti-S serum								
	Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A.....	+	—	—	+	+	—	+	+	—
" B.....	*+++	++	+	+	+	—	+	+	—
" C.....	+	+	—	*+++	++	+	++	+	—
Group X.....	+	+	—	+	+	—	*+++	++	+
Gran.....	+	+	—	+	±	—	++	+	—

* Typical "S" reaction.

(b) *Anti-R Sera*.—In anti-R sera, however, chemically decapsulated suspensions show a marked agglutination to a high dilution of serum and the reaction gives the characteristic appearance of R agglutination. The suspensions react equally well in all anti-R sera regardless of their type derivation. (Table IX.)

It is interesting to note that a closely related organism, *Encapsulatus granulomatis*, when subjected to the same conditions, is also agglutinated by anti-R sera. This explains in a measure the confusion experienced with allied encapsulated Gram-negative organisms. The species antigen of the Friedländer's bacillus is related to some extent to the species antigen of what have been considered allied organisms.

To recapitulate, encapsulated strains (Friedländer's bacillus) are transformed into "R" strains by heating in acid solution. The transformed cells react identically as "R" cells obtained by cultural methods. They agglutinate in anti-S sera depending upon the presence of species antibody, and in anti-R sera they behave as typical "R" strains.

TABLE IX.

Agglutination by Anti-R Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Anti-R sera	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	1:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	+++	++
	" C	+	++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	*Gran.	++	++	+	—	—
" B	Type A	+++	+++	++++	++++	+++
	" B	++++	+++	++	+	—
	" C	++	++++	++	+	—
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	++	+	—
Group X	Type A	++++	++++	++++	++++	+++
	" B	++++	++++	++	++	+
	" C	++	+++	++++	+++	++
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	+	—	—

* This organism was isolated from an infection of granuloma inguinale.

DISCUSSION.

"S" strains of Friedländer's bacillus are characterized biologically by mucoid growth in liquid media, by capsule formation, and by exalted virulence. As antigens they give rise in the serum to type-specific antibodies. Consequently, such an immune serum will agglutinate all organisms of the homologous type, afford passive protection in white mice against infection by strains of the same type, and precipitate the soluble specific substance derived from the type strains. The extent of species antibody in anti-S sera is negligible

provided immunization has not been intensive. The experience of former investigators, including our own, has been that in general prolonged immunization, even with capsule-bearing organisms, has yielded a serum so abundant in the species antibody as to obscure type-specific reactions. It is now recognized (7) in this connection that dissociation of the bacterial antigen occurring spontaneously *in vitro* and *in vivo* is a factor to be considered in immunization. That the dissociation observed in this study is due to a cleavage of the specific antigen complex in the animal body rather than a condition of cultural development is substantiated by certain evidence to be presented in a later paper.

"R" strains of Friedländer's bacillus on the other hand are characterized by loss of capsule formation, loss of the elaboration of soluble specific substance, and by attenuation of virulence. The sera of animals immunized to R strains contain only the species antibody. In other words anti-R sera agglutinate only capsule-free strains, irrespective of their type antecedent. Furthermore, anti-R sera fail to react with encapsulated organisms. They afford no passive protection against infections with virulent type-specific strains of Friedländer's bacilli, and they do not precipitate the soluble specific substance derived from organisms of homologous or heterologous type.

It is obvious, therefore, that the nature of antibody response in the animal is dependent upon the character of the bacterial cell which is utilized for immunization. Immunization with encapsulated strains engenders type-specific antibodies. On the other hand, a bacterial culture composed of both encapsulated and capsule-free cells, as is often the case, induces both the type-specific and species-specific antibodies. The predominance of the S or R component determines the predominance of the one or the other antibody. In either case confusing cross-agglutination reactions will be encountered, as they have been in the past, which are difficult of interpretation unless cognizance is made of the underlying principles. Immunization which is effected with cells devoid of capsules, as advocated by former workers (20, 21, 25), gives rise to only the species antibody which exhibits none of the type relationships. Similarly, in the agglutination reaction will be reflected the composition of a strain.

The studies on the cell constituents of *Pneumococcus* to which reference has already been made reveal a striking similarity in the immunological behavior of the two encapsulated races. There has been observed in both species the occurrence of encapsulated, virulent organisms which differ serologically and which undergo degradation to such an extent as to become capsule-free, avirulent, and serologically undifferentiated.

These principles find a remarkable analogy even among flagellated bacteria as is indicated by Smith and Reagh (29), Orcutt, and Felix. These authors have shown independently that certain differences are demonstrable in the serological behavior between flagellated and non-flagellated organisms of the same strain. These differences have been related to the presence of two antigens, the flagellar or ectoplasmic, and the somatic or endoplasmic antigen.

CONCLUSIONS.

1. "S" strains of Friedländer's bacillus produce capsules, soluble specific substance, and are of exalted virulence. "S" strains are type-specific and react with only the type-specific antibodies of the homologous types.

2. Immunization with "S" cells induces the formation of antibodies which agglutinate type specifically, precipitate the corresponding soluble specific substance, and protect white mice against infection caused by organisms of the same type.

3. "R" strains of Friedländer's bacillus produce no capsules, produce no soluble specific substance, and are not pathogenic. "R" strains are serologically undifferentiated from each other and react with only the species antibodies.

4. Immunization with "R" cells induces antibodies which do not agglutinate encapsulated organisms, do not precipitate soluble specific substance, and do not afford protection against infection by Friedländer's bacillus. Anti-R serum contains only the species antibody which reacts with any capsule-free organism regardless of its type origin.

5. Decapsulation of "S" cells by heat and acid chemically converts a type-reacting organism into a species-reacting organism.

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KIDNEY FUNCTION IN PNEUMONIA.

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INTRODUCTION.

Studies of the changes which occur in kidney function during the course of lobar pneumonia have been made by several investigators. The results which they have obtained show little uniformity, and the conclusions arrived at are quite at variance. Tileston and Comfort (1) studied 14 cases of lobar pneumonia and found an elevation in the blood urea during the febrile stage. They made 8 phenolsulphonphthalein tests, and found a diminution in excretion in 3 instances. Schwartz and McGill (2) in a series of 20 cases, obtained very similar findings. Longcope and Peters (3) studied 4 cases of pneumonia incidental to their study of renal function in serum disease. The phenolsulphonphthalein excretion was below normal in only one of their 6 determinations, and the blood urea was normal in all instances. They compared the urea in the urine with the urea in the blood by means of McLean's index, and found normal values in all their determinations. Bookman (4), on the other hand, found a diminished 'phthalein output in 5 of his 6 cases of pneumonia, and considered that without other signs of nephritis, most cases showed distinctly impaired function. The blood urea showed no striking change in his series. Lewis (5) investigated 8 cases of lobar pneumonia in his study of the clinical value of Ambard's "coefficient of urea excretion." He found a well marked increase in the phenolsulphonphthalein excretion, the average for 2 hours being 70.5 per cent, compared with 60.2 per cent in his series of 28 normal cases. The values given for the blood urea nitrogen were all normal, but the output of urea in the urine was higher than one would expect in a normal individual. Lewis points out that "any kidney capable of raising the concentration of the urinary urea to a level 80 times that in the

blood can scarcely be called a hyperpermeable organ," but that "the increased rate of output indicates an increased functional ability of the kidney."

Frothingham (6) failed to find a general increase in phenolsulphonphthalein excretion. Of 15 determinations done on 10 cases, only one (which was done during the febrile stage) showed an increased output (83 per cent), while decreased outputs were obtained twice. Five of the cases showed a McLean index higher than normal during the febrile stage, which returned to normal limits during convalescence in the four instances in which the test was repeated. The index was subnormal in 3 determinations, once during the disease, and twice after the patients were afebrile. This author concludes that since the increase in the index was not associated with an increase in phenolsulphonphthalein a hyperactivity of the kidney does not occur during fever. He also considered that the tests failed to show consistent evidence of impaired renal function during the course of the disease.

Of the six authors, therefore, three emphasize the finding of impairment of kidney function, two emphasize the absence of impairment, and one emphasizes the occurrence of hyperfunction during the course of lobar pneumonia. It seemed to us, therefore, that a series of observations, repeated at intervals during the course of the disease, might throw some light on the cause of such discordant results. The urea concentration index, recently described by Van Slyke, Linder, Hiller, Leiter and McIntosh (8), by means of which the concentrating power of the kidney for urea under standard conditions is estimated, seemed to be a convenient method for the study. It also seemed to us that such a study would be necessary before this test could be used as a diagnostic measure in suspected cases of nephritis, in which a febrile disease, such as pneumonia, was present, at the time of the test, or shortly antecedent to it.

METHODS.

In the material presented, the phenolsulphonphthalein was given intravenously, and the specimens collected at the end of one hour, and 2 hours, when this was possible. This object was not always achieved on account of the inability of the patients to void at the

proper time. Such results were discarded. It seemed to us that in view of the frequency of difficulty in voiding, phenolsulphonphthalein tests are not wholly reliable in such cases, unless catheterization is carried out. The urea concentration index is open to the same objection, but in this instance the error can be minimized by lengthening the period of urine collection. In this study, all urine collections which were open to suspicion of being incomplete were discarded. The patients were given 150 cc. of water fifteen minutes before the first voiding, and urine collections were made over a period of 2 hours as a standard procedure. The urine and blood urea was determined by the urease method of Van Slyke and Cullen (7) except in a few urine urea determinations, which were made by a gasometric method which will shortly be described by Van Slyke. The significance of the urea concentration index may be described by stating that it represents the number of times the kidneys concentrate urea in excreting it from the blood into the urine, when the urine volume output is at the average normal rate of 1 cc. per minute, or 1 cc. per hour per kilo of body weight. Values of 35 to 80 for the index are regarded as being normal, and 55 per cent as the lower limit of normal for phenolsulphonphthalein determination.

RESULTS.

In all, 13 cases were studied. The results are summarized in table 1. The most characteristic finding is to be noted in cases 1, 2, and 3. These show a normal index early in the disease, followed by a marked increase, which usually occurred before the crisis, and lasted in one case as long as seven days after it. The index subsequently returned to normal. In cases 4, 5, and 6 the same supra-normal indices were observed during the febrile period, and in each case, a return to normal occurred after the crisis. The phenolsulphonphthalein return showed the same supra-normal phase, and a good parallelism can be observed between it and the index in cases 1, 2, 3, and 4.

None of the other cases showed definitely supra-normal indices. Nevertheless, the same tendency to hyperfunction can be observed in several of them. Case 9 showed a series of three indices between the fourth and the tenth day, which were probably considerably above his individual normal level. This case, as well as cases 8 and 12, also showed an increased output of phenolsulphonphthalein.

TABLE 1.

Number of cases	Hospital number	Day of disease	Temperature	Phthalein output in 2 hours	Urine volume per hour	Blood urea nitrogen	Urine urea nitrogen	Urea concentration index	Remarks
			°F.	per cent	cc.	gm. per liter	gm. per liter		
1	5426	3	103	92.6	42.8	0.238	15.72	61.1	
		5	102.2		63.5	0.164	12.74	87.5	
		10	99	87.6	70	0.129	9.79	89.7	
		15	99		63	0.193	9.12	37.5	Crisis on seventh day
2	5422	3	103.8	67.2	67.5	0.253	16.27	75.4	
		4	102.4	83.4	34	0.222	21.37	80.1	
		7	100.3	88.7	53	0.126	9.78	80.7	Crisis on fifth day
		10	100.4	94.3	225*	0.137	2.56		Serum disease
		12	99.6	88.3	75.5	0.099	6.46	81	Serum disease
		16	98	81.8	35	0.159	12.68	67.4	
3	5442	5	104		85	0.165	5.24	45.4	
		9	100.5	82.7	37.5	0.121	8.95	70.2	Crisis on sixth day
		11	99	75.6	28.5	0.132	13.03	81.6	
		19	101	58.9	55	0.116	6.04	59.8	Serum disease
		25	103	80.1	22.2	0.098	7.53	56.1	Serum disease
4	5450	7	102	86.9	58.5	0.149	12.79	87.1	
		10	100	59.1	30	0.150	13.21	64	Crisis on seventh day
		14	100	63.9	55.4	0.108	4.65	42.5	Serum disease
5	5527	4	103.5		168.2	0.214	12.85	97	
		5	102	28	213	0.207	15.00	131.7	
		8	99	68.2	107	0.145	8.52	75.9	Crisis on fifth day
6	5542	6	104	42.1	100	0.136	10.58	103.2	
		14	99	73.9	60	0.172	8.66	51.7	Crisis on seventh day
7	5418	2	103		46.9	0.341	14.58	38.2	
		3	102	55.9	78.3	0.320	12.92	45.5	
									Died

*This urine is above the "augmentation limit" and the index cannot be calculated.

TABLE 1—*Concluded.*

Number of cases	Hospital number	Day of disease	Temperature	Phthalein output in 2 hours	Urine volume per hour	Blood urea nitrogen	Urine urea nitrogen	Urea concentration index	Remarks
			<i>°F.</i>	<i>per cent</i>	<i>cc.</i>	<i>gm. per liter</i>	<i>gm. per liter</i>		
8	5427	3	103	48.7	95	0.164	6.24	47.2	
		5	103.2	84.4	80	0.381	12.09	36.1	
		9	99.2	92.5	65	0.263	14.64	57.1	Crisis on sixth day
		15	99	83.7	67	0.243	8.40	36	
		18	99	66.1	85	0.156	4.92	37	
9	5436	2	99.5		77.5	0.572	11.07	21.5	
		4	101	65.9	48	0.229	17.94	68.5	Crisis on second day
		6	100.6	49.1	47.5	0.156	12.33	68.8	Mild serum disease
		10	98	79.4	46.5	0.176	13.83	67.6	
		12	98		60	0.162	8.58	51.8	
10	5607	4	103.5	32.8	100	0.173	6.95	51.8	
		9	102	50.3	86	0.405	5.36	15.8	
		14	99	59	71	0.240	7.27	32.9	Crisis on ninth day
		22	99	60.1	28.6	0.152	7.30	33.1	
11	5602	4	103.5		95.6	0.279	10.84	44.6	
		7	102		80	0.303	7.37	25.5	
		12	100	75	50	0.220	16.80	63.4	Crisis on ninth day
12	5661	4	103	84.2	62.5	0.332	12.88	39.7	
		8	99	87.7	42	0.209	14.91	59.8	Crisis on fourth day
		11	99.6	75.2	47.5	0.212	11.98	50.5	
13	5662	2	102	60.6	29	0.185	7.89	31.1	
		6	101	29.5	13	0.127	8.67	33.3	Lysis on fourth day
		9	100	69.4	29	0.110	6.39	42.4	

On the other hand, 4 cases were observed which showed a moderate decrease in the index of concentration. Cases 9 and 10 showed such a result on the day of their crises; in case 9, this was followed by a

relative increase in the index, whereas in case 10, the index failed to reach normal values in subsequent tests. In case 11, there was one subnormal index before the crisis occurred, and in case 13 one sub-

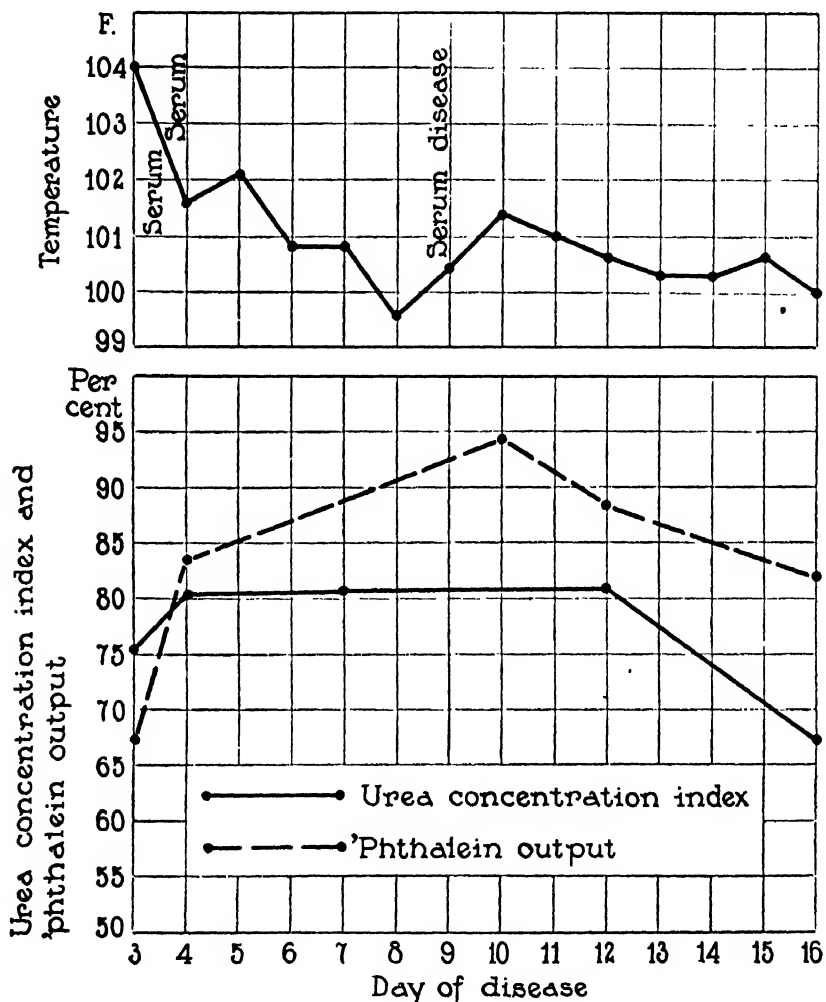


FIG. 1. CASE 2. Illustrating phase of renal hyperfunction after lobar pneumonia.

normal value before and one after the crisis. Both cases gave normal tests at a later stage of convalescence.

Definitely subnormal phenolsulphonphthalein returns are to be served in cases 5, 6, 8, 10, and 13, one such result being noted in each

case. These individual determinations are not in agreement either with the other phenolsulphonphthalein tests in the same patients, nor with the index found on the same day. One feels inclined to minimize the value of these determinations as a proof of decreased kidney function in these cases. On the whole, however, a moderately good agreement is to be observed between the phenolsulphonphthalein and the index, considering the fact that catheterization was not resorted to in order to obtain complete urine collections.

There are two factors which affect the blood urea level which deserve comment. The increased protein catabolism of the disease tends to raise the blood urea, and values of over 0.3 grams of urea N per liter are not unusual, even with perfectly normal kidney function. On the other hand, the hyperfunctioning kidneys tend to neutralize this tendency, and when hyperfunction occurs, the blood urea tends to be lower than when it does not occur.

Whether definite anatomical changes occur in the kidney, with which the increase or decrease in functional ability might be correlated, we are unable to state. Albuminuria of slight or moderate degree was present in all the cases during the febrile stage, and it seems reasonable to suppose that some degree of cloudy swelling of the renal epithelium might have been found. None of the cases had any history or clinical findings suggestive of coexistent nephritis.

The cases have been reviewed to observe whether the type of pneumococcus responsible for the pneumonia bore any relation to the changes found in kidney function. No such relation between type of infecting organism and the functional changes is to be observed. Six of the cases were diagnosed as Type I, and of these, 5 received antipneumococcus serum. Subsequently, large doses of neochinchophan were given to 4 of them. Four of the cases developed serum disease, manifested by urticaria. None of these incidents seem to have influenced kidney function in any way.

CONCLUSIONS.

1. During the course of lobar pneumonia, the kidneys frequently show a period of increased functional ability. This period usually begins before the crisis, and lasts until several days after it. In a series of 13 cases, the hyperfunction was shown by an increased urea

concentration index in 6 cases, by an increased phenolsulphonphthalein output in 7 cases, and by one or the other test in 9 cases.

2. A moderate depression of kidney function was observed in 4 cases; this depression was found sometimes before, and sometimes after the crisis.

3. Serious impairment of kidney function during pneumonia was not encountered in this group of cases.

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THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

IV. ON THE NATURE OF THE SPECIFIC POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS.

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It was shown in 1917 by Dochez and Avery (1) that there is present in fluid cultures of pneumococci a substance which precipitates specifically in antipneumococcus serum of the homologous type. This "soluble specific substance" was also found in the body fluids of the infected organism, and was even demonstrated in the urine of many patients suffering from pneumonia due to pneumococci of Types I, II, and III. Dochez and Avery also found that the specific substance is not destroyed by boiling, that it is readily soluble in water and precipitable from it by alcohol or acetone, that it does not dialyze through parchment, and that its serological reactions are unaffected by digestion with trypsin.

In later publications from this laboratory (2-4) it has been shown not only that this soluble specific substance appears to belong to the group of polysaccharides, but also that it is possible to isolate from cultures of each antigenic type of *Pneumococcus* a chemically distinct polysaccharide, different in many particulars from those of the other two types, and possessing in the highest degree the property of precipitating the antiserum to the type of *Pneumococcus* from which it was derived. Analogous sugar derivatives with specific properties have been isolated in the case of the Friedländer bacillus (Heidelberg, Goebel, and Avery (5), Mueller, Smith, and Litarczek (6)), the tubercle bacillus (Laidlaw and Dudley (7), Mueller (8)), and even from yeast (Mueller and Tomcsik (9)), and their presence has been shown in *Streptococcus viridans* (Lancefield (10)). The so

called "residue antigens" of Zinsser and Parker (11), first considered as containing in part protein degradation products or nucleic acid derivatives (12), appear also to be specifically reacting polysaccharides.

The immunological significance of this new type of sugar derivative, so frequently encountered among microorganisms, has been discussed elsewhere in papers from this and other laboratories (13-16), and its importance in the study of bacterial specificity demonstrated (17-19). It would accordingly be a matter of great interest to have some knowledge of the chemical structure of the new group of polysaccharides, and the present paper describes a beginning made in the case of the soluble specific substance of Type III pneumococcus.

As previously found (4), this substance may be obtained as a snow-white, amorphous powder, free from nitrogen and ash, and with marked acidic properties. It is precipitated from aqueous solution by strong mineral acids, and is then insoluble in water when dried. An aqueous solution of the sodium salt shows a specific rotation of about -34° and gives precipitates with many heavy metal salts and with barium hydroxide in excess. At a dilution as high as 1 to 6,000,000 it still yields a detectable precipitate with Type III anti-pneumococcus serum. The specific property disappears when the substance is hydrolyzed by means of acid. The products of hydrolysis are glucose and an acid which was considered to be a condensation product of a hexose and a -uronic acid.

The chief stumbling block to chemical progress with the bacterial polysaccharides has been the small amount of material available for investigation. It has been found possible in the case of the Type III pneumococcus to overcome this objection to some extent. In the first place this microbe produces far more specific substance than do the other two antigenic types of *Pneumococcus*, and in addition its polysaccharide is the easiest of the three to isolate and purify. Furthermore, once the properties of the Type III specific substance had been established, it was considered proper to add glucose to the usual *Pneumococcus* broth (2). In this way a greatly increased growth was obtained, and whereas the yields of specific substance were originally from 6 to 9 gm. per 300 liters of broth, they now rose to 35 to 40 gm. With these increased amounts of material it has been possible to show that the complex sugar acid mentioned above is not

only the chief product of hydrolysis, but that it is apparently the actual unit from which the whole polysaccharide is built up. The structure of the acid has also been partly elucidated.

EXPERIMENTAL.

A. Preparation of the Type III Soluble Specific Substance.

For the preparation of the specific polysaccharide the Type III pneumococcus was grown and the specific substance isolated as in Paper III (4), except that by the addition of 0.3 per cent of glucose to the liquid medium far greater growth was

TABLE I.

Preparation No.	Acid equivalent.	Specific rotation.	Total N.	Reducing sugars on acid hydrolysis.	Ash.	Precipitation with antipneumococcus serum.
33*	343	-30.5°	0.0	73.0	0.0	1:6,000,000
53†	338	-34.0°	0.0	70.0	0.0	1:6,000,000
55†	338	-33.0°	0.0	65.0	0.0	1:6,000,000

* Preparation 33 was isolated from glucose-free broth.

† Preparations 53 and 55 were obtained from the glucose broth.

obtained. Consequently the yield of purified polysaccharide was greatly improved, 300 liters of broth now furnishing between 35 and 40 gm. The material obtained was identical with that previously described, as will be seen from Table I.

B. Hydrolytic Products of the Type III Soluble Specific Substance.

1. *Products of Partial Hydrolysis.*—10 gm. of air-dried specific polysaccharide were dissolved in 40 cc. of 75 per cent (by weight) sulfuric acid at 0°. After standing for 21 hours in the ice box the solution was poured into 1 liter of water and the sulfuric acid quantitatively removed with barium hydroxide. The resulting solution was concentrated *in vacuo*. The product was found to be divisible into three distinct fractions. Fraction I was obtained by precipitation from a volume of 50 cc. with a slight excess of barium hydroxide saturated at 60°. Fraction III was also obtained as a barium salt by treating the concentrated, barium-free supernatant liquid from Fraction I at a volume of 20 cc. with 3 volumes of the same barium hydroxide solution. Fraction II represents the supernatant liquid of Fraction III, from which the remaining organic matter was precipitated with basic lead acetate after the excess of barium hydroxide had been removed. The lead was of course removed by means of hydrogen sulfide.

It was found possible further to subdivide Fraction I by precipitating a neutral-

ized solution with 10 per cent copper sulfate. From the precipitate (*Ia*), and the supernatant liquid (*Ib*), the free acids were obtained by eliminating the copper as its sulfide, the sulfate as barium sulfate, and concentrating to dryness. A summary of the properties of these five fractions will be found in Table II.

None of these fractions reacted specifically with Type III anti-pneumococcus serum. Since no glucose was found it is evident that the initial hydrolytic products of the Type III specific substance consist of a mixture of sugar acids probably of varying molecular weight, but containing a similar bionic acid unit as shown by the figures for the acid equivalent.

On further hydrolysis with boiling dilute acid these fractions were hydrolyzed to the disaccharide acid (aldobionic acid) described in a later section.

TABLE II.

Fraction No.	Acid equivalent.	$[\alpha]_D$	Reduction (calculated as glucose).
			<i>per cent</i>
I	340*	-12.6°	12.5
<i>Ia</i>	343	-11.0°	11.8
<i>Ib</i>	310	-7.9°	17.2
II	340	+9.2°	20.1
III	340	-8.3°	31.0

* This value remained unchanged in the presence of excess N/14 NaOH on the water bath.

2. *Products of Prolonged Hydrolysis.*—22.5 gm. of polysaccharide (containing 6.5 per cent of water of hydration) were slowly added with stirring and occasional cooling to 100 cc. of 75 per cent (by weight) sulfuric acid. After complete solution had resulted the mixture was placed in the ice box overnight and was then diluted to 2.7 liters and boiled 5 hours under a reflux. The sulfuric acid was removed quantitatively with barium hydroxide and the barium sulfate washed free from reducing sugars. The combined filtrates were concentrated to 150 cc. *in vacuo* and treated with an excess of basic lead acetate solution. After standing overnight in the cold, the portion precipitated by lead (Fraction I) was filtered off. The filtrate (Fraction II) was treated with hydrogen sulfide, and after the lead sulfide had been washed free from reducing sugars the filtrate was concentrated *in vacuo* and a small remainder of sugar acid (Fraction III) was removed by treating with an excess of basic lead acetate. All three fractions were now freed from lead, and in each case the lead sulfide was washed until free from reducing sugars. After

removal of the hydrogen sulfide the amount of sugar in each fraction was determined by the Shaffer-Hartmann method (20).

Fraction II, which had previously been shown to yield only glucose (4), contained 2.0 gm. of the sugar. Fractions I and III showed 8.7 and 0.2 gm., respectively, calculated as glucose. Since the two latter fractions contain a sugar, as will be shown later, having only one-half the reducing power of glucose their weights are ascertained by doubling the glucose value. On adding the three amounts thus obtained it is seen that 19.8 gm. of reducing sugars are accounted for, or 94 per cent of the theory. The total weight should be $22.5 \times 0.935 = 21.0$ gm.

a. Properties of Fraction I.—A portion of Fraction I was evaporated repeatedly with water *in vacuo* to remove acetic acid and was then reprecipitated as its lead salt. This was freed from lead and evaporated to complete dryness, yielding a friable, amorphous product. This material, which forms by far the major portion of the hydrolytic products of the specific polysaccharide, has been described in a preceding paper (4).

The acid equivalent of the crude disaccharide was determined by titration. 36.2 mg. neutralized 5.04 cc. of $N/50$ NaOH. This gives an acid equivalent of 361; calculated for $C_{11}H_{19}O_{10}COOH$, 356. A micro sugar determination by the method of Shaffer and Hartmann (20) on a sample of 1.92 mg. gave a back titration of 12.68 cc. of $N/200$ $Na_2S_2O_3$. This corresponds to copper and glucose equivalents of 2.02 and 0.95 mg., respectively. Reduction 49.5 per cent, calculated as glucose.

0.2882 gm. made up to 15 cc. with H_2O , $l = 2$, α , $+0.30^\circ$. $[\alpha]_D = +7.8^\circ$.

b. Formation of a Morphine Salt.—0.7 gm. of the dry sugar obtained as above was dissolved in 8 cc. of water and morphine was added in excess. Since the salt could not be made to crystallize from solutions containing water it was evaporated to complete dryness over phosphorus pentoxide and taken up in a mixture of equal parts of absolute methyl and ethyl alcohols. A small amount of dark insoluble gum was filtered off. During prolonged standing in the ice box a crystalline product gradually separated. After recrystallization from the same solvent the salt melted at $153\text{--}156^\circ$ and was difficultly soluble in the usual anhydrous solvents.

0.2154 gm. substance: 3.7 cc. N_2 at 23° , 760 mm.

0.1003 " " : 0.1968 gm. CO_2 and 0.0557 gm. H_2O .

Calculated for $C_{29}H_{39}O_{15}N$ ($C_{12}H_{20}O_{12} \cdot C_{17}H_{19}O_3N$). C 54.26 per cent, H 6.13 per cent, N 2.18 per cent

Found. C 53.52 per cent, H 6.22 per cent, N 2.03 per cent.

0.3888 gm. diluted to 15 cc. with water gave, in a 2 dm. tube, a rotation of -2.48° , changing after 24 hours to -2.80° , where it remained constant.

$[\alpha]_D = -47.9^\circ$, changing to -54.0° .

c. Properties of the Sugar Acid Recovered from the Morphine Salt.—The morphine salt as prepared above was decomposed by the addition of a slight excess of aqueous ammonia. The resulting crystalline morphine was filtered off and the

solution containing the sugar acid was twice precipitated with basic lead acetate. The acid was obtained by evaporating the lead-free product to complete dryness. It formed a snow-white, amorphous residue.

0.1053 gm. diluted to 15 cc. with water gave a rotation of $+0.14^\circ$ in a 2 dm. tube, $[\alpha]_D = +10.0^\circ$.

2 mg. of substance, analyzed by the micro method of Shaffer and Hartmann (20), gave a titration of 12.90 cc. of $N/200 Na_2S_2O_3$ (factor 0.9660, blank 19.90 cc.). Copper equivalent, 2.12 mg., glucose 0.99 mg.; reducing sugar calculated as glucose, 49.5 per cent.

0.0956 gm. substance: 0.1398 gm. CO_2 and 0.0494 gm. H_2O .

Calculated for $C_{12}H_{20}O_{12}$. C 40.45 per cent, H 5.66 per cent.

Found. " 39.85 " " " 5.77 " "

35.17 mg. of sugar neutralized 4.85 cc. of $N/50 NaOH$. Acid equivalent, calculated for $C_{11}H_{19}O_{10}COOH$, 356; found, 363.

Except for a possible slight difference in optical rotation the crude disaccharide acid corresponds very closely in its properties with the purified acid.

0.2640 gm. of substance, heated with 12 per cent hydrochloric acid and analyzed by a modification of Pervier and Gortner's method (21) required 5.75 cc. of 0.1 $N KBrO_3$, corresponding to 0.0276 gm. of furfural, or 10.5 per cent. Since glucuronic acid yields about one-third of the amount of furfural liberated by pentoses under corresponding treatment (22), this figure would correspond roughly to the presence in the molecule of about 50 per cent of an acid of the glucuronic type.

The substance has marked acidic properties. It has one-half the reducing power of glucose and gives a strong naphthoresorcinol test. The acid is appreciably soluble in hot ethyl and methyl alcohols, and in hot glacial acetic acid, but fails to dissolve in the other usual organic solvents. On further prolonged hydrolysis it yields glucose. It thus appears to be built up from glucose and a -uronic acid in such a way that one reducing group remains free, and hence differs from any non-nitrogenous, naturally occurring disaccharide derivative hitherto described. Of all known sugar derivatives, it would seem most closely related to desaminochondrosin, the nitrogen-free anhydro derivative of chondrosin, a component of chondroitin sulfuric acid, which, in turn, occurs in mucoproteins (23).

d. Oxidation of the Sugar by the Method of Willstätter and Schudel (24).—0.1450 gm. of the sugar acid (recovered from its morphine salt), when analyzed by the method of Willstätter and Schudel, reduced 8.14 cc. of $N/10$ iodine. Theoretically 1 cc. of $N/10$ iodine should oxidize 0.0178 gm. of the disaccharide acid. 8.14 cc.

therefore correspond to 0.1448 gm. of disaccharide acid. The sugar is obviously quantitatively oxidized and its reducing group must be aldehydic in nature since this method is specific for aldoses. The substance may, therefore, be termed an aldobionic acid.

e. Oxidation of the Aldobionic Acid with Strong Nitric Acid.—1.5 gm. of specific polysaccharide were dissolved in 8 cc. of 75 per cent sulfuric acid at 0°. After standing overnight in the ice box the solution was diluted with water until the acid was of normal concentration and was boiled 5 hours under a reflux. The aldobionic acid was isolated over the lead salt as described above. At a volume of 4 cc. its aqueous solution was treated with 10 cc. of 1:1 nitric acid, allowed to stand 15 hours, boiled for 3 minutes, and then quickly evaporated on a large watch-glass over a boiling water bath. No formation of mucic acid could be observed, so the solution of the oxidation product, at a volume of 6 cc., was made strongly alkaline with 40 per cent potassium hydroxide and then acidified with an excess of glacial acetic acid. It was then seeded with a small crystal of potassium acid saccharate and allowed to stand at 0° for 24 hours. The crystals which had formed were filtered off, washed with a few drops of ice water, and dried. 0.20 gm. was obtained. The salt was recrystallized from 1 cc. of boiling water, 0.100 gm. of pure potassium acid saccharate being recovered.

0.0496 gm. dry substance: 0.0173 gm. K_2SO_4 .

Calculated for $HOOC(CHOH)_4COOK$. K 15.75 per cent.

Found. K 15.65 per cent.

f. Hydrolysis of the Disaccharide with Acid.—1.0 gm. of the dry aldobionic acid was dissolved in 50 cc. of normal sulfuric acid and boiled 20 hours under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide and the filtrate concentrated *in vacuo* and boiled with norit. The clear colorless solution, at a volume of 30 cc., was treated with an excess of basic lead acetate to remove the unaltered aldobionic acid. The filtrate was treated with hydrogen sulfide, filtered, and concentrated to dryness *in vacuo*. The residue was diluted to 20 cc. An analysis by the Shaffer-Hartmann method showed it to contain 0.1980 gm. of reducing sugars calculated as glucose. In a 2 dm. tube the solution gave a rotation of $+1.07^\circ$. $[\alpha]_D = +54.1^\circ$. The remaining solution was treated with 3.5 mols of phenylhydrazine acetate and heated for 1 hour on the water bath. The entirely crystalline osazone which was formed was filtered off and washed with a few drops of methyl alcohol. The yield was 0.10 gm. The product melted at 203–204°. The initial specific rotation was -54.5° , mutarotating to -30° after 48 hours.

From the melting point of the osazone, its direction of mutarotation, and finally from the specific rotation of the sugar solution itself, it is justifiable to conclude that this product of the hydrolysis of the aldobionic acid is glucose, and that the hexose half of the molecule is, therefore, glucose. The other half of the molecule (the sugar acid)

is largely destroyed by acid hydrolysis (*cf.* (22)). Whether the saccharic acid identified in the preceding section arises from oxidation of the glucose or of the -uronic acid, or both, cannot be stated as yet.

g. Oxidation of the Aldobionic Acid with Bromine.—2.4 gm. of the acid were dissolved in 50 cc. of water and to the solution were added 8 gm. of barium carbonate and 1 cc. of bromine. After 2 days 0.5 cc. more bromine was added. 4 days later the solution was filtered, the excess of bromine blown out with air, and the barium and hydrobromic acid removed quantitatively with sulfuric acid and silver sulfate. The resulting solution, freed from silver and sulfuric acid, showed the presence of 10 per cent of unaltered disaccharide by its reduction value. Thus 90 per cent of the aldobionic acid had been oxidized to the corresponding dibasic acid; *i.e.*, the free reducing group had been oxidized.

It was thought possible to hydrolyze the dibasic acid, but boiling with normal sulfuric acid showed only a slight increase in reducing sugars. It may be, of course, that hydrolysis actually took place with the subsequent decomposition of the aldehydic sugar acid. It has as yet been impossible to isolate in a state of purity either the oxidation product itself, or the products of its hydrolysis. That the -uronic acid portion of the molecule is still intact, however, is indicated by the fact that the oxidation product gives a strong naphthoresorcinol test.

DISCUSSION.

In the original communication on the soluble specific substance of Type III pneumococcus (3) it was pointed out that on hydrolysis a product with some of the properties of glucuronic acid was obtained. In a later paper (4) it was shown that glucose was one of the products of hydrolysis, while the other isolated corresponded not with glucuronic acid itself but with a more complex derivative of the glucuronic type, possibly consisting of glucuronic acid combined with a hexose.

The following is now presented as evidence, when considered collectively, that this portion of the hydrolytic products of the Type III specific substance, precipitable by basic lead acetate, is actually a compound of glucose and a hexose-uronic acid.

1. The reducing power of both the crude and the purified anhydrous substance is 50 per cent of that of glucose.

2. The acid equivalent is found to be 363, while the value calculated for $C_{11}H_{19}O_{10} \cdot COOH$ is 356.

3. As an acid, the substance forms a morphine salt which can be crystallized and purified by recrystallization. The analysis of the

salt gives values for carbon, hydrogen, and nitrogen checking closely with the theoretical. The purified acid, recovered from the salt, is scarcely different from the crude material.

4. The prolonged hydrolysis only a small amount of glucose, in addition to unhydrolyzed material, can be isolated, the acid half of the portion hydrolyzed apparently decomposing similarly to glucuronic acid.

5. The reducing group of the disaccharide acid is aldehydic, as shown by the fact that it may be quantitatively determined by the Willstätter-Schudel method. The substance also gives the color reaction with naphthoresorcinol characteristic of the glucuronic acid type.

Since the easily isolable mucic acid is not formed on hydrolysis and oxidation with nitric acid, the acid portion of the molecule can scarcely be galacturonic acid. The saccharic acid actually recovered from the oxidation mixture certainly arises at least in part from the glucose fraction of the molecule; whether the acid portion takes part in its formation or gives rise to some other soluble acid must be left for future work to determine.

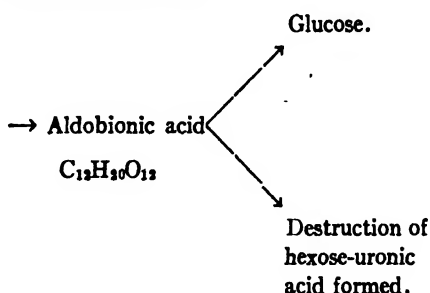
As to the position of the union of the glucose to the sugar acid, the evidence at hand does not permit any conclusion. The linkage may be either through the reducing group of the glucose, or through the reducing group of the sugar acid. That the union is glucosidic is indicated by the fact that on further hydrolysis the reducing power of the aldobionic acid increases to about 65 per cent before dropping owing to destruction of the hexose-uronic acid liberated.

If an analysis be made of the quantitative data obtained on the hydrolysis of the original polysaccharide (p. 479), it becomes evident that the aldobionic acid accounts for about 85 per cent of the total products of hydrolysis, while only 9.5 per cent is glucose, and 5.5 per cent unaccounted for. Thus by far the major portion of the polysaccharide seems to be constructed from molecules of this disaccharide acid. Now it has also been found (p. 481) that the aldobionic acid itself slowly hydrolyzes, to the extent of about 1 per cent per hour, on boiling with dilute mineral acid. It is therefore not illogical to assume that the 9.5 per cent of glucose liberated during the hydrolysis of the polysaccharide owes its origin, not to a separate

part of the carbohydrate molecule, but chiefly to a secondary reaction involving the aldobionic acid. This assumption is all the more justified by the fact that no glucose is split off during the preliminary hydrolysis by 75 per cent sulfuric acid in the cold. Since, also, these partial hydrolysis products show, by their acid equivalents, one carboxyl group for every two sugar nuclei, it would seem that the polysaccharide as a whole is built up of units of the aldobionic acid.

The condensed or polysaccharide form of a hexose-hexose-uronic acid should have the formula $(C_{12}H_{20}O_{12})_n - (n - 1)H_2O$, or $(C_{12}H_{18}O_{11})_n$. A substance of this composition should have an acid equivalent of 338 and a carbon and hydrogen content of 42.6 per cent and 5.4 per cent respectively. These figures are practically identical with actual analytical values obtained ((4) p. 733, Preparation 33 II. Acid equivalent, 340; C, 42.7 per cent; H, 5.3 per cent). Thus one may justifiably conceive of the Type III soluble specific substance as a condensation product of the disaccharide acid, $C_{12}H_{20}O_{12}$, built up in such a way that the carboxyl groups remain free. Hydrolysis by means of acid follows the course:

Polysaccharide acid \rightarrow Intermediate acids



In view of the evidence collected it is believed that the specific polysaccharide of the Type III pneumococcus is a definite chemical individual composed of units of a difficultly hydrolyzable aldobionic acid in which glucose and a hexose-uronic acid are combined in such a way that one aldehydic group and the carboxyl remain free. The polysaccharide is thus unusual not only in its possession of immunological specificity, but in its chemical constitution as well.

The question as to whether its unusual structure bears any relation to its immunological properties must be left for future work on this and other specific polysaccharides for a decision.

SUMMARY.

1. The soluble specific substance of Type III pneumococcus is shown to yield on hydrolysis a small amount of glucose and chiefly a disaccharide acid of a type not hitherto observed in any non-nitrogenous polysaccharide.

2. The disaccharide acid corresponds to the formula $C_{12}H_{20}O_{12}$ and contains one carboxyl group and one aldehydic reducing group in the molecule. It yields a crystalline morphine salt and appears to consist of 1 molecule of glucose condensed with 1 molecule of a hexose-uronic acid through one of the two reducing groups.

3. The specific polysaccharide is believed to be built up of units of this aldobionic acid, and thus to belong to a new type.

In conclusion the writers wish to express their gratitude to Dr. P. A. Levene for his many helpful suggestions, and to Dr. W. A. Jacobs for his assistance as well.

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The volume of each apparatus, and thus its constant, was obtained by the method of Warburg (3). Brodie's fluid was used in the manometer. The volume occupied by the egg contents in the vessel, *i.e.* the whole egg minus the air sac, was assumed to be equal to the weight of the egg minus the weight of the shell (approximately 6 gm.). In general, eggs of the same size and shape were selected.

A control was made with each test; at first in the form of a fertile egg of 1 day incubation, but later, when this was found unnecessary, with a vessel empty except for the alkali. The experiments were done in a constant temperature room, eliminating thereby the use of a water bath.

A thermometer ground into each glass cover registered the temperature, which averaged approximately 39.0°C. The small fluctuations which did occur were not found to affect appreciably the results. Moreover, it was found that the large surface of alkali exposed provided for maximum absorption of CO₂ without the necessity of shaking. Shaking for a minute prior to reading the manometer made no difference in the result. Nor was it found, when thin rubber tubes in which cold water flowed were run along one side of the vessel to cool the wall over this area and thereby to initiate by convection a regular circulation, as in Barach's (4) recently constructed human oxygen chamber, that any acceleration of CO₂ absorption took place.

About $\frac{1}{2}$ hour (the length of time being judged by the behavior of the control) was allowed for conditions to reach equilibrium. After this time readings were taken at varying intervals during periods of 2 to 6 hours until repetition of approximately similar results made one confident of their reliability. During the intervals between tests, the manometer and vessel were connected with an oxygen bag, so that the concentration of oxygen within the vessel remained always the same.

To obtain values for the rate of oxygen absorption per gm. of body weight the following figures are necessary, (1) the constant for the vessel (previously calculated); (2) the manometer readings, (3) the weight of the whole egg, and (4) the weight of the embryo.

One phenomenon was observed which we have not been able to explain. The embryo of an incubation age over 16 days, even when connected with the oxygen bag, did not survive in the apparatus over 12

hours. Their metabolism after 3 to 6 hours gradually fell. If the vessel was fully opened to the air for a few minutes the embryo would revive. Apparently it had nothing to do with a lack of oxygen, accumulation of CO_2 , or changes in the humidity; neither was there an accumulation of ammonia.

TABLE I.
Metabolism of Chicken Embryos as a Function of Age.

1	2	3	4	5	6	7	8	9	10	11
Age.	No. of observations.	By experiment. O_2 per day per gm. wet weight.	Standard deviation. \pm	From curve. O_2 per day per gm. wet weight.	O_2 per day per gm. dry weight.	Solid oxidized per day per gm. dry weight.	Solid stored per day per gm. dry weight.	Absorption per day per gm. dry weight.	CO_2 per day per gm. wet weight.	Resp. quot.
days		cc.		cc.	cc.	gm.	gm.	gm.	cc.	
6	8	50.0	2.1	50.0	896	0.444	0.665	1.11	29.9	0.60
7	5	38.4	2.3	43.0	735	0.364	0.584	0.95	29.6	0.69
8	3	36.7	1.5	39.0	628	0.311	0.510	0.82	29.3	0.75
9	10	39.2	1.3	36.5	562	0.278	0.478	0.75	29.0	0.79
10	7	32.8	1.2	35.0	500	0.248	0.465	0.71	28.5	0.81
11	7	33.9	1.2	34.0	442	0.219	0.465	0.68	28.0	0.82
12	9	33.9	1.2	33.2	378	0.187	0.465	0.65	27.0	0.81
13	5	32.4	0.7	32.5	322	0.159	0.465	0.62	25.7	0.79
14	5	30.3	0.6	31.7	259	0.128	0.447	0.57	24.0	0.76
15	7	34.3	1.3	30.5	209	0.103	0.395	0.50	22.0	0.72
16	11	29.0	1.4	28.7	175	0.087	0.320	0.41	20.1	0.70
17	5	26.1	1.0	26.2	152	0.075	0.250	0.33	18.1	0.69
18	6	21.1	1.3	23.2	131	0.065	0.215	0.28	16.2	0.70
19	3	20.5	0.3	20.0	113	0.056			14.2	0.71

Column 6 = figures calculated by the aid of values for the percentage of solid substance previously determined (Murray (1)).

Column 7 = values in Column 2 divided by 2019.3 (amount of oxygen absorbed when 1 gm. of fat is burned).

Column 8 = figures previously obtained (Murray (1), Table III).

Column 10 = figures read from smooth curve previously obtained (Murray (5)).

RESULTS.

The results of the oxygen determinations (Table I) may be seen (Fig. 1) to demonstrate a decrease in metabolic rate per gm. of body weight with age. This conclusion confirms that reached when the carbon dioxide was determined; except that in a more precise analysis

and comparison of the results it appears that the oxygen estimations show a sharp fall of metabolism during the first days of the period under observation, whereas the carbon dioxide figures do not (5). As has been mentioned in the introductory remarks, however, numerous complications arising from the variability of unknown factors cast doubt upon the value of CO_2 elimination as a measure of catabolic change.

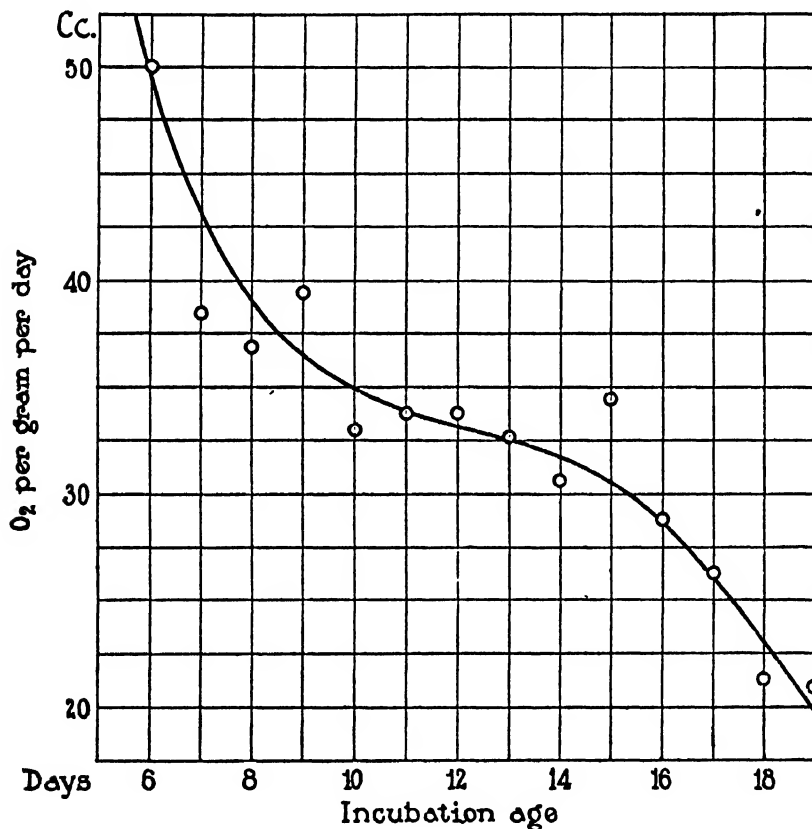


FIG. 1. Oxygen consumption in cc. per gm. of wet weight of chick embryo per day, as a function of age.

The oxygen determinations on the other hand were well controlled and presented no obvious factor to vitiate their use as indices of metabolic activity.

To oxidize 1 gm. of fat approximately 2000 cc. of O_2 are absorbed; whereas to oxidize 1 gm. of protein (966.3 cc.) or starch (828.8 cc.) about 900 cc. of O_2 are used (6). The total oxygen consumption for

the first 19 days, estimated by graphical integration, comes to 2988 cc., which on the basis that only fat is burned during incubation leads to the conclusion that 1.48 gm. of dry substance (*i.e.* fat) is oxidized during that period. If only protein and starch were burned, it would require over 3.28 gm. to use the observed amount of oxygen. Previous chemical analyses have shown that approximately 1.62 gm. of substance is burned during the first 19 days, a figure which may now be accounted for on the assumption that 92 per cent of the metabolism is oxidation of fat, and the rest of protein and carbohydrate. This value is to be compared to 98 per cent fat oxidation found by measuring the CO₂ output. The former figure is probably more accurate.

During the last 5 days of incubation, when about four-fifths of the total oxidation takes place, the respiratory quotient is approximately 0.71, which points to fat consumption, during this period. The earlier values for the respiratory quotient are somewhat higher (up to 0.81); but they are variable and it is uncertain whether they deserve consideration. The results point to some error during the first 3 days when the CO₂ figures, and thus the quotient, also seem to be definitely too low.

If we discard the carbon dioxide estimations in favor of these later O₂ determinations and assume as we may without undue error that catabolism is at the expense of fat, we arrive at some notion of the changes in the metabolic rate with age.

Regarding the organism energetically and dynamically, the amount of energy exchange measures its activity or vitality. Hence, the amount of energy stored plus the amount set free might be used as a criterion of aliveness. By adding the rate of storage in terms of weight (previously obtained) to the rate of elimination, likewise in terms of weight as measured by oxygen usage, one obtains the desired value; namely, the rate of dry mass absorption per gm. of body weight per day (Table I). It may be seen (Fig. 2) that there is a marked fall with age in the rate of absorption expressed in these terms. Reasons have been enumerated for believing that during the first half of incubation, when the amount of metabolism is small relative to the total metabolism during incubation but large relative to the weight of the embryo, there is a not inappreciable amount of protein and carbohydrate oxidized. If this were a fact a straight line rather than an S-shaped curve as graphically represented might be indicated.

The consumption of food during the early days is enormous. On the sixth day for instance the embryo absorbs over its own mass of dry substance. Assuming that the water content of the diet is ap-

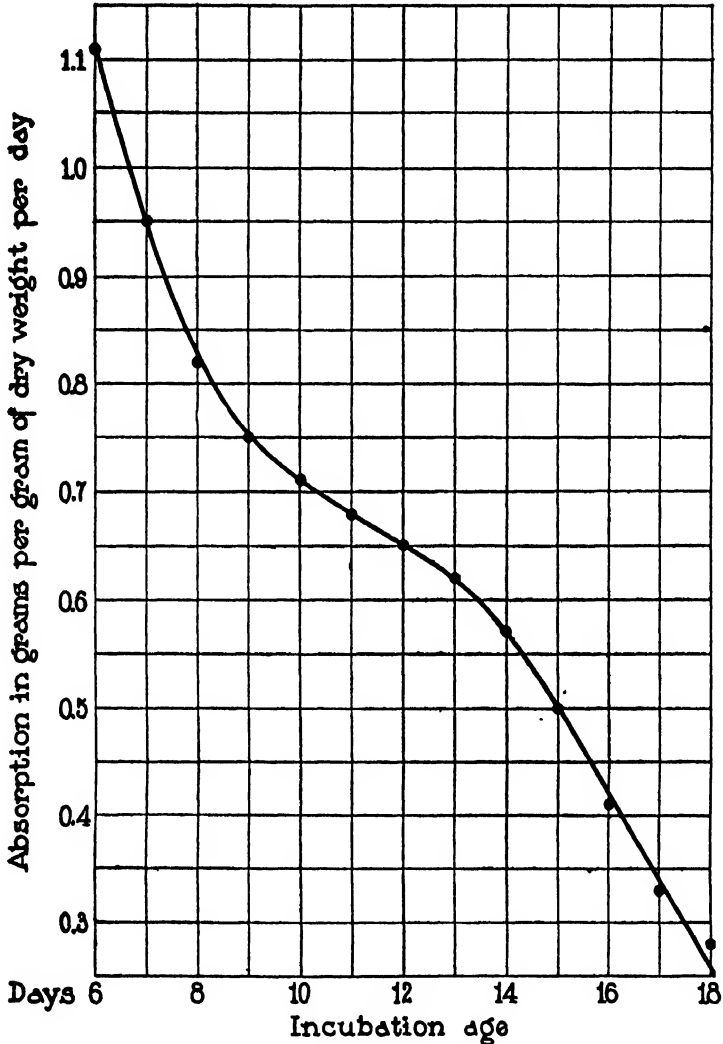


FIG. 2. Absorption of solid matter in gm. per gm. of dry weight of chick embryo per day, as a function of age.

proximately that of the tissues, this would be equivalent to a mature man eating about 150 pounds of food per day. During the 12 days under observation, however, the percentage rate of absorption falls

to about 25 per cent (one-fourth its earlier value). According to Lotka, a mature meadowlark consumes about 6.6 per cent of its own weight a day (7) which would suggest a fall in absorption rate during the postembryonic period of a degree comparable to that which occurs during the 12 days before hatching.

SUMMARY.

1. The previous findings that the rate of metabolism per gm. of body weight decreased with age, and that during the incubation period catabolism was mostly at the expense of fat, have been confirmed.

2. These determinations of the rate of oxygen uptake have afforded more precise values for the catabolic rate and thus permit estimations of the changes with age in the rate of absorption.

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IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF ENCAPSULATUS PNEUMONIÆ
(FRIEDLÄNDER'S BACILLUS).

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A previous publication (1) on the biological classification of Friedländer's bacillus reported the existence of at least three specific types and a heterogeneous group among these bacilli. The groupings, designated as Types A, B, and C, and Group X, are sharply defined and highly specific by a number of immunological reactions. In a later communication (2) it was stated that encapsulated strains are usually virulent, produce soluble specific substance, and as antigen, induce the formation of type-specific antibodies which operate effectively both in test-tube reactions and in animal protection tests. Capsule-free strains, on the other hand, are avirulent, do not produce soluble specific substance, and as antigen stimulate only the undifferentiated species antibody. Type-specific antisera react irregularly with capsule-free strains; and the species-specific antisera, while reacting with capsule-free organisms regardless of type derivations, do not react at all with encapsulated cells.

The accumulated evidence on the serological reactions of Pneumococcus (3) and Friedländer's bacillus (1, 2, 4, 5) discloses that both species are composed of specific types which are referable to the elaboration of soluble specific substance by the organisms. Under certain conditions, the cells degrade into capsule-free bacteria which, among other changes, show lack of virulence and capsule formation, loss of elaboration of specific carbohydrate, and loss of type-specific antigenicity—all of which properties are the opposite of those which characterize their encapsulated antecedents. In virtue of the striking parallelism in the immunological behavior of Pneumococcus and Friedländer's bacillus it seemed of interest to project into the latter group the principles which govern the immunological relationships of the cell constituents of Pneumococcus.

The comparative immunological studies of the soluble specific substance and the nucleoprotein of *Pneumococcus* (3) reveal that the soluble specific substance, a nitrogen-free carbohydrate, reacts specifically with antipneumococcus serum of the homologous type. In the dissociated, dissolved, state, it does not serve as antigen; but in the form in which it exists in the cell it functions antigenically to produce type-specific antibodies. The nucleoprotein, on the other hand, is protein in nature and induces in the animal an antiserum which contains only the antiprotein or common, species antibody.

The present study is concerned with the immunological relationships of cell constituents of Friedländer's bacillus and the occurrence of these constituents in culture and body fluids of infected animals.

Methods.

The Soluble Specific Substance.—Methods for the fractionation of soluble specific substance of Friedländer's bacillus have been described in papers from this laboratory (5). It was shown at that time that carbohydrate derived from Strain E (Type B) is dextrorotatory, shows an acid equivalent varying from 670 to 716, is nitrogen-free, and on hydrolysis yields about 75 per cent reducing sugars. It reacts only in type-specific sera to a dilution of 1 to 4 million. Purified, nitrogen-free polysaccharides of Types A and B prepared in this laboratory were utilized in the present study through the courtesy of Drs. Heidelberger and Goebel.

The Nucleoprotein.—Several methods were employed for the separation of the protein and none of the methods were entirely satisfactory. The yield was usually small and the solutions underwent denaturation on keeping. The method finally adopted, however, made use of non-encapsulated cells (since no difference could be shown between the protein derived from encapsulated and capsule-free cells, respectively). The growth from the surface of the agar in Blake bottles was washed off in sterile distilled H_2O . To this suspension NaOH was added to an ultimate concentration of .005 N. The suspensions were frozen and thawed successively a dozen times or more and then diluted 3–4 times with distilled H_2O and centrifuged. The resultant supernatant was filtered, so that a sterile cell-free filtrate was obtained. Precipitation was then effected with a minimum amount of N acetic acid and the precipitate was whirled down. The supernatant was discarded and the precipitate was redissolved in a minimum amount of .01 N NaOH. Usually acid precipitation and solution with alkali were repeated and the final product was made up in .85 per cent NaCl. All protein solutions were standardized on the basis of nitrogen content.

Immunological Reactions.—The method of immunization, the reactions of agglutination and precipitation and protection test have been described in an earlier paper (1).

EXPERIMENTAL.

I. The Soluble Specific Substance.—(a) *Antigenic Properties.*—Repeated observations already published from this laboratory leave no doubt that the chemically purified polysaccharide of *Pneumococcus* is non-antigenic. Similar studies (6) of Zinsser, Mueller, and their associates also record the lack of antigenicity of “residue antigen” from a number of bacterial species. The “residue antigen” of these investigators is a substance which is extracted from bacteria and which bears a definite relation to the specific character of the bacterial cell.

In the present study observations on the antigenicity of the carbohydrate of Friedländer's bacillus are confined to the immunization of rabbits with the polysaccharide derived from Group X. In this instance a solution of bacterial cells was prepared from an encapsulated strain and it consequently contained dissociated soluble specific substance. Immunization with this product even in the presence of nucleoprotein yielded no specific antibodies, as will be pointed out later. Since the results from various sources indicate that bacterial polysaccharides are not antigenic, the lack of specific antibody response to a solution containing both protein and carbohydrate is evidence of similar conditions in the case of Friedländer's bacillus, also. At the same time it is clear that bacterial dissolution is accompanied by antigenic dissociation.

(b) *Serological Properties.*—It has been previously demonstrated (1, 2, 4, 5) that the polysaccharides derived from Friedländer's bacillus react specifically with immune sera of the homologous type. (Cf. in this connection Table IV.) In fact, sufficiently conclusive proof has been presented to show that just as has been shown with *Pneumococcus*, the soluble specific substance confers upon the cell its immunological type specificity.

II. The Nucleoprotein.—(A) *Antigenic Properties.*—It is realized that the acetic acid-precipitable material represents more than the nucleoprotein of the bacterial cell, and that it is a mixture of proteins rather than a single antigenic unit. Nevertheless, for the purposes of the present study, this fact offers no difficulty in either the performance or interpretation of the various reactions employed. Rab-

TABLE I.

Agglutination of "S" Strains of Friedländer's Bacillus by Anti-P Sera.

Antigen encapsulated strain	Anti-P sera derived from									Anti-S sera—Type			
	Type A			Type B			Group X			A	B	C	X
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A.....	—	—	—	—	—	—	—	—	—	++++	—	—	—
" B.....	—	—	—	—	—	—	—	—	—	—	++++	—	—
" C.....	—	—	—	—	—	—	—	—	—	—	—	++++	—
Group X.....	—	—	—	—	—	—	—	—	—	—	—	—	++++

++++ indicates complete, disc agglutination.

* The figures represent dilution of serum.

TABLE II.

Agglutination of "R" Strains of Friedländer's Bacillus by Anti-P Sera.

Anti-P sera	Protein antigen from	Final dilution of serum							Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Type A	Type A	++++	++++	++++	++++	++++	++++	++++	—
	" B	++++	++++	++++	++++	++++	++	++	—
	" C	++++	++++	+++	+++	++	+	+	—
	Group X	++++	++++	++++	++++	++++	++++	++++	—
" B	Type A	++++	++++	++++	++++	++++	+++	++	—
	" B	++++	++++	++++	++++	++++	+++	++	—
	" C	++++	++++	++++	+++	+++	++	++	—
	Group X	++++	++++	++++	++++	++++	++++	++++	—
Group X	Type A	++++	++++	++++	+++	++	+	+	—
	" B	++++	++++	++++	++++	++++	+++	++	—
	" C	++++	++++	+++	+++	++	++	+	—
	Group X	++++	++++	++++	++++	++++	++	+	—

++++ indicates complete agglutination; +++, marked agglutination, ++, moderate agglutination; +, slight agglutination.

bits were immunized to the protein isolated from representative strains of Types A and B and Group X. The immune sera were tested for agglutinins, precipitins, and protective antibodies.

(a) *Agglutinins*.—Antiprotein sera do not contain type-specific

agglutinins for encapsulated strains of Friedländer's bacillus. Evidence for this statement is found in Table I where it is seen that antisera prepared by immunization with protein derived from serologically different strains do not react with the encapsulated cell of either homologous or heterologous type.

It will be recalled that capsule-free (R) strains of Friedländer's bacillus (2) stimulate the formation of the common, undifferentiated

TABLE III.

Agglutination by Anti-P Sera of Suspensions of Friedländer's Bacillus Decapsulated by Porges' Method.

Anti-P serum derived from	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	2:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	++++	+++
	" C	++	+++	+++	++	+
	Group X	+++	+++	++++	++++	+++
	Gran.*	++	++	+	—	—
" B	Type A	++	+++	++++	++++	++++
	" B	++++	++++	++++	+++	++
	" C	++	+++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	Gran.	++	++	+	—	—
Group X	Type A	+	++	++++	++++	++++
	" B	++++	++++	++++	+++	+
	" C	+	+	+++	++	++
	Group X	++	+++	++++	++++	+++
	Gran.	++	++	++	+	—

* This organism is a strain of granuloma bacillus—isolated from an infection of granuloma inguinale.

species antibody; but that they are unable to provoke type-specific antibodies. Accordingly, it seemed pertinent to determine the reaction of antiprotein sera on the non-encapsulated strains. The results of the reactions are given in Table II. It is seen that capsule-free cells derived from any of the serologically different types agglutinate equally well in all the antiprotein sera. The agglutination is

characteristic of the R cells (2) and occurs at a high dilution of serum. Antiprotein sera, in other words, behave in this respect similarly to anti-R sera. It was to be expected, then, that anti-protein sera would agglutinate, also, suspensions of Friedländer's bacilli after decapsulation by Porges' method. Earlier observations (2) pointed out the serological similarity of capsule-free strains obtained by cultural and chemical means. Table III reveals that suspensions of encapsulated cells treated as Porges recommends are agglutinated in antiprotein sera just as are the "R" strains.

TABLE IV.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-P Sera.

Serum	Soluble specific substance of Friedländer's bacillus											
	Type A						Type B					
	2	20	50	100	250	500	2	20	50	100	250	500
Type A (P).....	-	-	-	-	-	-	-	-	-	-	-	-
“ B “.....	-	-	-	-	-	-	-	-	-	-	-	-
“ C “.....	-	-	-	-	-	-	-	-	-	-	-	-
Group X “.....	-	-	-	-	-	-	-	-	-	-	-	-
Type A (S).....	-	+	++	++	+	-	-	-	-	-	-	-
“ B “.....	-	-	-	-	-	-	++	++	++	++	++	++
Normal.....	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates compact disc precipitation with clear supernatant; ++, marked disc precipitate; +, thin film-like scale; -, ground glass turbidity.

The figures represent dilution in thousands.

(b) *Precipitins*.—It has been shown that type-specific precipitins are induced only by the encapsulated cell. Added confirmation of this fact is derived from the observation that antiprotein sera do not react with the specific polysaccharides of Friedländer's bacillus. This is evident from the results presented in Table IV. It is definite that none of the antiprotein sera are able to cause precipitation of carbohydrate isolated from strains of Type A or B.

That antiprotein sera react with capsule-free strains of Friedländer's bacillus constitutes direct evidence of the presence of the species antibody. That the species antibody is in reality an antiprotein

TABLE V.

Precipitation by Anti-P Sera of the "Nucleoprotein" of Friedländer's Bacillus and Allied Organisms.

Antigen*	Anti-P serum from	Ultimate dilution of protein						
		200	1000	2000	4000	8000	16,000	32,000
Type A protein	Type A	+++	+++	++	++	+	±	—
	" B	+++	+++	++++	+++	+++	+++	++
	Group X	+++	+++	++	+	—	—	—
		500	2500	5000	10,000	20,000	40,000	80,000
Type B protein	Type A	++++	++++	+++	++	+	±	—
	" B	++	++	+++	+++	+++	+++	+
	Group X	+++	+++	++	+	±	—	—
		200	1000	2000	4000	8000	16,000	32,000
Type C protein	Type A	++	++	++	+	+	±	—
	" B	++	++++	++++	++++	+++	+++	+
	Group X	—	+	++	++	+	—	—
		500	2500	5000	10,000	20,000	40,000	80,000
Group X protein	Type A	++++	++++	+++	++	+	±	—
	" B	++	++	+++	+++	++	++	±
	Group X	++	++	+	+	±	—	—
		500	2500	5000	10,000	20,000	40,000	80,000
<i>B. aerogenes</i> protein	Type A	++	+	+	±	—	—	—
	" B	++	+	+	±	—	—	—
	Group X	++	+	±	—	—	—	—
		300	1500	3000	6000	12,000	24,000	48,000
<i>B. coli</i> protein	Type A	++	+	+	±	—	—	—
	" B	++	++	+	+	±	—	—
	Group X	++	++	+	+	—	—	—
		200	1000	2000	4000	8000	16,000	32,000
Granuloma bacillus protein	Type A	++	++	+	+	—	—	—
	" B	++	++	++	+	+	—	—
	Group X	+	+	±	—	—	—	—

++++ indicates heavy precipitation, supernatant clear; +++, marked precipitation with cloud; ++, marked cloud, no precipitation; +, cloud; ±, faint cloud.

* None of the proteins were precipitated by normal serum.

antibody is evidenced by the reaction of protein precipitation in antiprotein sera. The results of these reactions are presented in Table V. It is seen that sera prepared against protein isolated from three serologically different strains cause the precipitation of protein derived from any of the four different strains. It becomes obvious therefore that nucleoprotein induces the formation of species antibodies which cause agglutination of capsule-free cells and precipitation of protein and that the reactions exhibit none of the type relationships.

In addition, Table V reveals that antiprotein sera of Friedländer's bacillus reacts with protein derived from *B. aerogenes*, *B. coli*, and granuloma bacillus. Stated in another way the protein of Fried-

TABLE VI.

Protection Offered by Anti-P Sera against Infection with Friedländer's Bacillus (Type A).

Type A encapsulated culture	Antiserum derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 20	.2	D. 19	
.0001	.2	" 16	.2	" 15	
.00001	.2	" 39	.2	" 16	D. 24
.000001	.2	" 39	.2	" 43	" 39
.0000001					" 65

D. indicates death, the numerals representing the number of hours before death occurred.

länder's bacillus bears a definite serological relationship to proteins of closely allied species. This lends considerable assistance in the interpretation of the results of former investigators who have observed that anti-Friedländer sera caused agglutination of *B. rhinoscleromatis* (7-10), *B. aerogenes* (11), typhoid (12), and granuloma bacillus (13), etc. The explanation of such cross-agglutinations appears to depend upon the fact that immunization with non-encapsulated strains or prolonged immunization with encapsulated strains stimulates the formation of agglutinins which act not only on R cells

of Friedländer's bacillus, but on R cells of closely related species, as pointed out above.

III. Protection.—Immune sera prepared by immunization with encapsulated Friedländer's bacilli confer upon white mice specific protection against infection by strains of the homologous type (1).

TABLE VII.

Protection Offered by Anti-P Sera against Infection by Friedländer's Bacillus (Type B).

Type B encapsulated culture	Anti-P serum derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 16	.2	D. 15	
.0001	.2	" 16	.2	" 15	
.00001	.2	" 39	.2	" 20	D. 16
.000001	.2	" 39	.2	" 67	" 39
.0000001					" 39

D. indicates death, the numerals representing the number of hours before death occurred.

TABLE VIII.

Precipitation of the Nucleoprotein of Friedländer's Bacillus by Anti-S Sera.

Anti-S Friedländer sera	Friedländer protein derived from							
	Type A		Type B		Type C		Group X	
	*600	6000	1200	12,000	900	9000	500	5000
Type A.....	++	+	+	++	++	+	++	+
" B.....	+	—	+	—	±	—	+	—
" C.....	++	+	++	+	++	+	++	+
Group X.....	+	—	++	—	+	±	+	—

* These figures represent the dilution of protein.

Immunization with non-encapsulated cells, however, yields no protective antibodies (2). Protective substances, therefore, accompany type-specific antibodies. It was anticipated then that lacking type-specific agglutinins and precipitins, Friedländer antiprotein sera would afford no passive protection against active infection. That this is

actually the case is borne out by the data given in Tables VI and VII. The lack of any protective action by antiprotein sera against infection with virulent strains of homologous and heterologous types is striking.

(b) *Serological Properties.*—

1. *Precipitation of Protein in Anti-S Sera.*—Anti-Friedländer sera resulting from immunization with encapsulated strains are dominantly type-specific. Consequently anti-S sera contain negligible amounts

TABLE IX.

Precipitation by Anti-R Sera of the "Nucleoprotein" of Friedländer's Bacillus.

Antigen	Anti-R serum from	Dilution of protein						
		200	1000	2000	4000	8000	16,000	32,000
Type A protein	Type A	+++	+++	++	++	+	—	—
	" B	+++	++	++	+	—	—	—
	Group X	++	+	+	±	—	—	—
Type B protein		500	2500	5000	10,000	20,000	40,000	80,000
	Type A	+++	++	+	—	—	—	—
	" B	++	++	+	—	—	—	—
Type C protein	Group X	++	+	+	—	—	—	—
		200	1000	2000	4000	8000	16,000	32,000
	Type A	++	+	+	—	—	—	—
Group X protein	" B	++	+	+	—	—	—	—
	Group X	++	+	+	±	—	—	—
Group X protein		500	2500	5000	10,000	20,000	40,000	80,000
	Type A	+++	++	+	—	—	—	—
	" B	++	++	+	—	—	—	—
Group X protein	Group X	+++	+++	++	+	—	—	—

of antiprotein as has been pointed out previously (2) by the agglutination of R cells in anti-S sera. In virtue of the presence of antiprotein, such sera may cause precipitation of protein. This fact is illustrated in Table VIII. Type A anti-Friedländer serum definitely contains the species antibody, while the remaining type sera contain traces or none at all.

2. *Precipitation of Protein in Anti-R Sera.*—Capsule-free strains of Friedländer's bacillus are agglutinated in antiprotein sera (Table

II). Evidence of the reciprocal nature of this reaction was obtained in the precipitation of protein by anti-R sera. It is seen from the results presented in Table IX that anti-R sera cause the precipitation of protein from Friedländer's bacillus of the different types. The conclusion can be drawn, therefore, that the common species antibody reacts with the undifferentiated antigen of Friedländer's bacillus whether the antigen is in the form of non-encapsulated cells or in the form of dissolved protein.

TABLE X.

Precipitation of Friedländer Protein by Anti-P Sera after Adsorption of the Species Antibody by R Strains.

Antigen protein derived from	Anti-P serum (Type A) after adsorption by R strains derived from encapsulated strains of											
	Type A			Type B			Type C			Group X		
	*1:200	1:2000	1:8000	1:500	1:5000	1:10,000	1:200	1:2000	1:8000	1:500	1:5000	1:8000
Type A.....	—	—	—	—	—	—	—	—	—	—	—	—
" B.....	—	—	—	—	—	—	—	—	—	—	—	—
" C.....	—	—	—	—	—	—	—	—	—	—	—	—
Group X.....	—	—	—	—	—	—	—	—	—	—	—	—

* The figures represent the dilution of protein.

(c) *Precipitation of Protein in Antiprotein Sera.*—It has been shown above that protein derived from any of the serological types is precipitated in all antiprotein sera. (Cf. Table IV.)

Adsorption of Antibodies in Antiprotein Sera by Non-Encapsulated (R) Strains of Friedländer's Bacillus.

Because agglutination of R cells occurs in antiprotein sera, and precipitation of protein is obtained in anti-R sera, experiments were conducted to gain information concerning the identity of the antibody involved in both reactions. Each antiprotein serum was adsorbed with heat-killed suspensions of R cells derived from encapsulated strains of Types A, B, and C, and Group X. Adsorption was continued until all the agglutinins were removed for the adsorbing

strain. It was found that removal of agglutinins by one strain adsorbed the agglutinins for the remaining R strains also. Moreover, the loss of agglutinins by adsorption was accompanied by a loss of precipitins. A typical protocol is presented in Table X. It is clear that adsorption of a serum resulting from immunization with protein derived from a strain of Type A, for example, with any of the four "R" strains deprives the serum of precipitins for protein derived from any of the types. It appears therefore that the undifferentiated species antibody is the same antibody whether it occurs in anti-R or antiprotein sera.

TABLE XI.

*Occurrence of Soluble Specific Substance in Culture Filtrate.
Friedländer Bacilli.*

Strain	Dilution of filtrates											
	After 4 hrs.				After 8 hrs.				After 12 hrs.			
	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50
Type A.....	+	-	-	-	+	+	-	-	+++	++	+	-
" B.....	-	-	-	-	±	-	-	-	+	+	-	-
" C.....	+	-	-	-	++	+	-	-	++	++	+	-
Group X.....	±	-	-	-	++	+	-	-	++	+	-	-

*Occurrence of Soluble Specific Substance in Culture Filtrates of
Friedländer's Bacillus.*

It has been shown by Dochez and Avery (14) that the soluble specific substance of Pneumococcus is demonstrable in culture filtrates, and that the progressive increase of the carbohydrate bears a striking relation to the growth curve of the culture. Their results show clearly that the soluble specific substance is a product of metabolic activity rather than a product of cell disintegration.

In similar fashion, the polysaccharide of Friedländer's bacillus is demonstrable in actively growing cultures. Cell-free filtrates obtained at different intervals during the growth are precipitated specifically by anti-Friedländer sera. The data submitted in Table XI show that in some instances specific carbohydrate is present in filtrates as early as 4 hours after growth has been initiated. The

amount of the carbohydrate increases rapidly so that after 24 hours' growth a definite reaction may be obtained in culture filtrate in dilution of 1:20, and in one case (Type A) even in a dilution of 1:50.

Occurrence of Protein in Culture Filtrates of Friedländer's Bacillus.

The type specificity of anti-Friedländer sera depends in part upon the integrity, and the absence of R cells in the culture used for immunization. It becomes of importance, therefore, to determine the rapidity of the disintegration of Friedländer's bacilli. Dissociation may be estimated by the presence of the common or protein antigen. Accordingly an analysis of the protein content of culture filtrates of Friedländer's bacillus was made by the usual protein pre-

TABLE XII.

Occurrence of Protein in Culture Filtrates of Friedländer Bacilli.

Strain	Dilution of filtrates											
	At 12 hrs.		At 24 hrs.		At 72 hrs.		5 days		9 days			
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:5	1:10	1:20
Type A.....	-	-	-	-	-	-	-	-	+	±	-	-
" B.....	-	-	-	-	-	-	-	-	++	+±	+	-
" C.....	-	-	-	-	-	-	-	-	++	+	±	-
Group X.....	-	-	-	-	-	-	-	-	++	+	±	-

cipitation test. As brought out in Table XII, no protein was demonstrated in culture filtrates after 5 days' growth. On the 9th day, precipitation of protein was obtained in all the filtrates studied. In contrast to the carbohydrate which is elaborated during the period of active growth, the protein is demonstrable after this period and when cell disintegration takes place.

This fact becomes of great significance in immunization with suspensions of Friedländer's bacillus. Despite the fact that both disintegration and R cells are absent in the cultures used for immunization, anti-Friedländer sera may contain variable amounts of protein antibody. This is evidence that the body defenses not only engender type-specific antibodies, but also include a mechanism which causes a cleavage or disintegration of the specific antigen.

Occurrence of Soluble Specific Substance in Friedländer Infections.

The soluble specific substance of *Pneumococcus* has been demonstrated in the serum and urine of patients during pneumonia by Dochez and Avery (14). Blake (15) has shown that this is also a fact in pneumonia due to Friedländer's bacillus. This is the only reference of its kind concerning Friedländer infection which has come to our attention. In the present study experiments were performed to detect specific carbohydrates in rabbits infected by intraperitoneal injections of Friedländer's bacilli. The urine and blood of the infected rabbits were collected and tested for soluble specific substance

TABLE XIII.

Occurrence of Soluble Specific Substance in the Blood and Urine of Animals Infected with Friedländer's Bacilli.

Type of infection	Body fluid	Dilution of fluid		
		1:1	1:5	1:10
Type B	Serum	++	+	—
	Urine	+	—	—
" C	Serum	+++	++	+
	Urine	++	+	—

The precipitin reaction was obtained only in homologous immune serum.

by the usual precipitin technique. It is seen from Table XIII that the specific polysaccharide of Friedländer bacilli is present in both urine and serum of rabbits, and is demonstrable within 18 hours after infection.

DISCUSSION.

The soluble specific substance of Friedländer's bacillus endows the cell with type specificity, and is separable from the bacterial cell as a pure, nitrogen-free polysaccharide. When dissociated from the cell, it does not function as antigen, but in the form in which it exists in the cell it stimulates the formation of antibodies which cause type-specific agglutination of encapsulated cells, precipitate the carbohydrate derived from organisms of the homologous type, and afford

passive protection in white mice against infection by bacilli of the same type. As reacting substance, it is precipitated only in antisera resulting from immunization with encapsulated strains from which it is derived.

The nucleoprotein is separable from dissolved Friedländer's bacilli by precipitation with acetic acid in the cold. This constituent differs in nature and in serological behavior from the soluble specific substance. It is protein in nature and is a common, undifferentiated constituent of all types. It is antigenic and provokes in the animal the common protein or species antibody. The species antibody does not react with encapsulated bacilli of any type nor with the soluble specific substance of either homologous or heterologous types, and does not protect against infection with Friedländer's bacillus. Anti-protein sera, however, cause agglutination of capsule-free cells derived from any of the serological types by either cultural or chemical methods; and they react also with protein from all types. Moreover, the protein antibody is of a sufficiently general nature to react with protein from allied bacteria. In this fact resides the explanation for the confusing cross-agglutination reactions obtained with related organisms by former workers. That the protein antibody is of more or less common occurrence among other species of bacteria gains evidence from the results of numerous investigators. The studies from this laboratory show this with *Pneumococcus*, and the work of Lancefield (16), Hitchcock (17), and Tunncliffe (18) discloses a distinct serological relationship between various species of the Gram-positive cocci. The contributions of Dopter (19) and Eberson (20) depict similar relationships among the Gram-negative cocci, and Smith and TenBroeck (21) and Felix (22) offer comparable data for members of the typhoid-colon group.

Antisera prepared by immunization with protein or with a degraded non-encapsulated R culture contain antibodies which are identical in their immunological reactions.

The presence of soluble specific substance in filtrates of growing cultures suggests that it is a product of growth activity of the cell. The presence of protein in filtrates of old cultures only, indicates that it is a product of cell autolysis and disintegration.

CONCLUSIONS.

1. The soluble specific substance of Friedländer's bacillus is non-antigenic when dissociated from the cell. It is different for each type and it is highly reactive in the corresponding anti-S serum.

2. The nucleoprotein is antigenic, induces the species or protein antibody which reacts with capsule-free cells and protein derived from all types. Antiprotein sera do not react with either the encapsulated cell or the polysaccharide derived from it, and they offer no protection against infection.

3. Anti-R and antiprotein sera are identical in their behavior.

4. The carbohydrate of Friedländer's bacillus is demonstrable in filtrates of actively growing cultures and in the blood and urine of infected animals.

5. The protein is demonstrable in filtrates of only old, disintegrating cultures.

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A FILTERABLE VIRUS PRESENT IN THE SUBMAXILLARY GLANDS OF GUINEA PIGS.

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PLATE 33.

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During recent years much attention has been paid to peculiar alterations in the morphology of certain cells in lesions associated with the presence of filterable viruses. These changes are especially constant and well marked in the lesions of spontaneous and experimental herpes simplex. Indeed, they occur so constantly in this condition that Goodpasture and Teague (1) have used the presence of these cells as a guide in tracing the passage of the virus through the tissues of the infected animal. The uniformity of the cellular changes in herpes simplex and the constancy with which they occur renders this the most satisfactory of the virus diseases to be used as an example in discussing analogous and related conditions. Cells derived from the endothelium, epithelium, or mesenchyme may show these changes and although there may be variations in the appearance of the alterations in the different cells, on the whole they are very similar, whether the cells involved be connective tissue cells, epithelial cells of the cornea, or large ganglion cells of the central nervous system.

The characteristic features of these changes, as seen in tissues stained with eosin and methylene blue, are the following. The nucleus takes on a vesicular character and the limiting membrane is deeply stained with the basic dyes. Usually the inner surface of the membrane is irregular, as though the basic staining material were collected in granules or small clumps. A few of these granules are much larger than the others and these are considered to represent the nucleoli of the unaltered nucleus. Within the nucleus, usually at the center, is a round or oval body which stains either faintly or, more often,

deeply with acid dyes. The depth of staining depends to some degree, however, upon the length of exposure. The red-staining material may occupy not more than half, or even less, of the nuclear space or it may occupy almost the entire nucleus. In any case it is sharply limited from the surrounding nuclear material by a clear unstained halo. The material itself is usually granular, never hyaline or dense; it appears as though composed of a multitude of very fine granules compressed into a ball or mass.

Nuclear changes which cannot at present be differentiated from those occurring in the lesions of spontaneous and experimental herpes simplex also occur in the skin lesions of herpes zoster, in the skin lesions of varicella, and in the lesions experimentally produced in rabbits by the Virus III of Rivers and Tillett (2). Nuclear changes resembling to some extent those occurring in herpes simplex are also found in a variety of conditions, especially diseases of animals, such as, epidemic encephalomyelitis of horses, or Borna's disease, fowl-pox, certain diseases of fishes, etc.

Except for a few reported instances, nuclear changes like those occurring in herpes simplex have so far been found only in conditions in which the presence of a filterable virus has been demonstrated or in which the association of a virus of this group is very probable. The experimental production of these lesions except by the injection of filterable viruses has, in our hands, been unsuccessful. It is true that Luger and Lauda (4) have mentioned the occurrence of similar structures in a case of salvarsan dermatitis. But even though these lesions should be present in isolated instances of this kind, it would be necessary to demonstrate the absence of a filterable virus in the given instance before the present conception of the direct relationship between these nuclear changes and filterable viruses would become untenable.

Lipschütz (3) has considered that these nuclear changes represent "nuclear inclusion bodies" in the sense of Prowazek. He has collected all the conditions in which nuclear inclusions occur, and also the conditions in which unusual bodies or structures are found in the cytoplasm of the cells, into a great group of virus diseases, the causative agent of which he classes with the *Chlamydozoa-strongyloplasma*. Lipschütz has maintained that the bodies or structures seen within the nucleus represent a specific reaction of the cells to a living virus.

The bodies are not considered to be masses of parasites but are held to represent reaction products, associated with which is the virus.

This conception of Lipschütz has not been universally accepted, however. Luger and Lauda (4) who have devoted much study to the nature of these nuclear changes, maintain that they do not represent "inclusion bodies" in the sense of Prowazek, but that they are the result of a non-specific type of nuclear degeneration, which these authors call "oxychromatic degeneration." They refer to the observations of Heidenhain, who described two varieties of nuclear, chromatic substance, basic chromatin and oxychromatin. During nuclear degeneration occurring under certain conditions, the oxychromatin tends to collect in the center of the nucleus and the basic chromatin at the periphery. According to these writers the typical nuclear changes seen in the lesions of herpes simplex represent the final stages in this separation of oxy- and basic chromatin.

At the present time the evidence in favor of either of these views is not convincing. Our own observations suggest that the inclusion bodies are produced by the accumulation of a finely granular material which at first is scattered throughout the nucleus. This material which, in its scattered form, is very faintly acidophilic, takes on a deeper and deeper red stain as it accumulates into a mass, until the typical nuclear changes become manifest. The earlier stages of this process, however, are difficult to detect and usually only the fully developed nuclear "inclusion body" is seen. Until further knowledge concerning the chemical nature of these structures is obtained, it is not important, at least in the present connection, to decide whether we shall speak of "nuclear inclusions" or of "oxychromatic degeneration" of the nucleus. It is very important, however, to know whether these changes represent characteristic lesions due to the action of filterable viruses or whether they represent a form of degeneration which occurs under a great variety of conditions producing a non-specific injury to the cell. The burden of evidence at present points to the former concept, for it would be very surprising, in view of the careful study which has been made of cellular changes under various pathological conditions, that this striking nuclear alteration should have been frequently overlooked.

In a few isolated instances, pathologists have reported finding peculiar nuclear changes in human pathological material. VonGlahn and Pappenheimer (5) have recently collected the reports of sixteen human cases, and have added a case of their own, in which, at autopsy, in various viscera, cells with striking nuclear abnormalities were present. While the descriptions of these nuclear changes in the individual cases differ to some extent, in most instances they are sufficiently alike to justify the conclusion that the various writers were dealing with identical or closely related conditions. All of the cases reported, except that of VonGlahn and Pappenheimer, were in infants under 1 year, five of these were still-born. Five of them probably suffered from congenital syphilis, in one nephritis was present, in one pneumonia, one had "green stools," edema of feet, and bronchitis, another one hydrocephalus and focal interstitial nephritis. The case of VonGlahn and Pappenheimer was that of a male aged 36 years, who suffered from an abscess of the liver. Certain cells in the intestines, liver, and lungs were found to be of very large size, measuring at times 25 micra in diameter. Some of these were multinucleated. Within the nuclei of these cells were large acidophilic masses. In the lung these large cells were usually in continuity with the epithelium, but cells of granulation tissue and of the blood vessels also contained the nuclear inclusion masses.

In addition to these cases collected by VonGlahn and Pappenheimer, L. Jackson (6) has reported finding an ameba-like organism in the kidneys of a child. This child, 15 months old, suffered from diphtheria and died suddenly. At autopsy lesions of bronchopneumonia were found. In the kidney tubules were large cells which the writer interpreted as amebæ. It seems from the description that these may have been structures similar to those present in the cases collected by VonGlahn and Pappenheimer. Indeed, Jackson stated that the structures were similar to those described by Ribbert (7) and by Smith and Weidman (8), whose cases were included by VonGlahn and Pappenheimer in their series.

In most of the earlier human cases the nuclear inclusions were thought to be due to the presence of parasites, amebæ, or sporozoa, in the later cases they were described as nuclear degenerations. One of the cases in infants was reported by Goodpasture and Talbot (9) in 1921. These writers drew attention to the similarity between the nuclear changes in this case and the nuclear changes in the epithelial cells in varicella which were first described in 1906 by Tyzzer (10). They also called attention to peculiar cellular changes present in the epithelial cells of the salivary glands of guinea pigs which were first described by L. Jackson in 1920. Goodpasture and Talbot studied these cells in guinea pigs and concluded that the condition was an example of cellular transformation similar to that occurring in the lesions of infancy. They thought that there was no evidence that these cellular and nuclear changes were due to some intracellular infection. Von Glahn and Pappenheimer were the first to fully identify the large cells occurring in human cases with the abnormal cells met with in herpes simplex and related conditions. They regarded the intranuclear masses seen in their own case as nuclear inclusions, "identical in their morphology and staining reactions with the

bodies seen by previous observers in the viscera of infants, and by Lipschütz and others in tissues of spontaneous and experimental herpes, and in various neural and visceral lesions produced by the herpetic and related viruses." These writers were unable to carry on any experimental study, but offer the suggestion that the lesions in this case may have been related to the presence of an unknown virus.

Cellular Changes in the Submaxillary Glands of Guinea Pigs.

Unusual changes in the submaxillary glands of guinea pigs were first described by L. Jackson (11) under the title, "An intracellular protozoan parasite of the ducts of the salivary glands of the guinea pig." These structures were found in 26 of 48 pigs examined. She interpreted them as protozoa, probably coccidia. We have had no difficulty in confirming the observation of Jackson that in the ducts of the submaxillary glands of guinea pigs there are found unusual and striking structures which on first observation suggest a parasitic origin. However, we have been unable to differentiate the different stages of development resembling those of protozoa which Jackson described. The structures we have observed, however, conform in all particulars with those shown in the illustrations in Jackson's paper and there can be no doubt that we are dealing with the same abnormalities. We have examined the submaxillary glands of 75 guinea pigs over 6 months of age. Sections from these glands have been stained in eosin and methylene blue, and in 63 of the glands, or 84 per cent, these unusual structures have been found in larger or smaller numbers. From our own study we have identified them as swollen epithelial cells (Fig. 1). The nucleus of each of these cells contains a mass of granular material which is definitely acidophilic. The altered cells are found chiefly in the ducts of the serous portion of the gland, though, in a few instances, they have been seen in the mucous portion. The cells lie either on the basal membrane contiguous to the unaltered epithelial cells or within the lumen of the duct, evidently having pushed forward during the process of hypertrophy. The altered cells occasionally are not more than twice the usual size (Fig. 2) but in most instances they are much larger than this, up to 40 micra in diameter. The large size and red staining of the nuclear inclusions render them easily visible under the low power of the microscope. In ducts cut obliquely or longitudinally, not infrequently six or eight, or even more,

somewhat swollen but they did not equal in size the very large cells in the glands of full grown guinea pigs. Most of the transmission experiments, however, have been made by injecting the material in other locations in young pigs where these cells do not occur, such as the testicle, brain, lung, and tongue. The most striking results were obtained when the injections were made into the brain and in most of the experiments this has been the site of inoculation.

Minor modifications of the technique of the brain injections were made in certain experiments and the results were not always identical but the following description is typical of this experimental method and of the results obtained. A full grown guinea pig is killed and the submaxillary glands removed under sterile precautions. A small piece is removed from each gland and placed in Zenker's solution, to be later examined in order to make certain that typical lesions are present. Aerobic and anaerobic cultures of the submaxillary gland are made at this time to exclude the possibility of bacterial infection. The remainder of the glands is cut into small pieces with scissors and ground in a mortar in about 2 cc. of Locke's solution. This is then centrifuged at low speed for a few minutes, to remove the large particles, and the supernatant fluid is used for injection. 0.1 cc. of this suspension is then injected directly into the brain¹ of a guinea pig less than 1 month old. 24 hours following this injection the temperature of the injected pig is usually not elevated and the animal appears normal. After 48 hours, however, the temperature frequently becomes elevated, 105–106°, but without the animal showing any marked symptoms. On the 3rd day the guinea pig appears sick, the hair is raised, the animal fails to move about in the cage, and the temperature continues elevated. On the 4th day, the symptoms have become more marked. The animal now begins to show signs of heightened nervous irritability as indicated by tremors and slight convulsive movements. On the 5th day it is usually very ill, has irregular jerking movements, is unable to rise when placed on its side, and death ensues. When the brain is removed no gross abnormalities beyond congestion are seen. Cultures are made to rule out the possibility of bacterial infection, and the brain is placed in Zenker's solution.

¹ Guinea pigs were anesthetized with ether before injection.

Later when microscopic sections are examined there is found well marked exudate over the surface of the entire brain, including the cerebellum (Fig. 4). The exudate consists chiefly of mononuclear cells, lymphocytes, and large cells with vesicular nuclei. There is considerable edema and the blood vessels of the membranes are distended and filled with blood. In contrast with the lesions resulting from intracerebral infection of the brain with herpes simplex virus, very slight if any changes can be detected in the brain itself, the blood vessels of which appear normal. The most striking feature is the presence in the meningeal exudate of large numbers of cells, each of which contains an acidophilic mass within the nucleus. These cells resemble in all particulars the cells containing nuclear inclusion bodies which occur in herpes simplex and related conditions (Fig. 5). The number of abnormal cells present in the guinea pig lesion is much greater than the number usually present in the lesions following injection of herpes simplex virus or Virus III of Rivers. In no case have very large cells like those found in the submaxillary gland been found. 54 young guinea pigs have received intracerebral inoculations of an emulsion of the submaxillary gland of full grown guinea pigs. Most of these animals showed symptoms similar to those described, though in some the symptoms were delayed. The animals died or were killed at various times from the 2nd to the 12th day following the injection. In 48 of the guinea pigs, or 89 per cent of those injected, lesions as described above were found.

Emulsions of the submaxillary gland of full grown guinea pigs have also been injected into the testicles of sixteen young guinea pigs. In all cases a histologic examination of the submaxillary gland was made to make certain that the specific lesion was present in the material used for injection. 0.1 cc. of the emulsion was usually injected into each testicle. The inoculated guinea pigs showed some elevation of temperature for several days, but they developed no other symptoms. The animals were killed at various intervals from 4 to 10 days following the injection, and both testicles were removed. Histologic examination was later made of one testicle from each animal and in almost all cases some degree of cellular infiltration was found. In twelve instances, or in 75 per cent of the animals inoculated, cells containing typical nuclear inclusion bodies were present. These

were either in cells of the interstitial tissue, or in cells of the tubules, or in both.

Injections of an emulsion of the submaxillary glands of full grown guinea pigs were also made into the tongues of young guinea pigs. These animals developed no symptoms. They were killed and the tongues were removed on the 3rd, 5th, and 9th days following the injection. Microscopic study of these tongues showed a localized cellular reaction in the stroma, and cells containing typical nuclear inclusion bodies were present. A tongue removed on the 9th day after inoculation showed the most marked cellular infiltration. In no instance was the epithelium of the tongue involved.

Inoculations were also made into the lungs of three guinea pigs. One of these animals showed a rise of temperature on the 7th day, and was killed. The lung showed no gross changes but on microscopic examination a circumscribed mononuclear cellular infiltration was found. In a few of the alveoli in the involved area, large mononuclear cells containing typical nuclear inclusion bodies were present. Two other guinea pigs inoculated in the lungs were killed on the 11th and 22nd days after injection. Although a mononuclear cell infiltration was present in the lungs, no cells with nuclear inclusion bodies were found. The injection of emulsions of submaxillary glands of full grown guinea pigs into young guinea pigs therefore has quite regularly resulted in the production of a subacute inflammatory reaction and the appearance of cells containing nuclear inclusion bodies morphologically identical with those seen in herpes simplex and allied conditions.

Control Experiments.—These experiments suggest that the submaxillary glands of full grown guinea pigs contain a virus which is responsible for the reaction in the young guinea pigs, and it seems probable that the large cells and the reaction seen in the older pigs is a manifestation of natural infection with this virus. This view is supported by the following observations. Four young guinea pigs were inoculated in the brain with an emulsion of the submaxillary glands of other young guinea pigs, presumably tissue containing no large cells with nuclear inclusions. Subsequent microscopic examinations showed that there were actually no lesions in these glands. The results of these inoculations were entirely negative. Nevertheless, it seemed possible that the reaction observed in the tissues of young guinea pigs

following the injections of the glands of old guinea pigs was of a non-specific nature, and that any glands of external secretion might contain irritating substances which on injection into the brains of young guinea pigs might give rise to an inflammatory reaction, and that under these conditions cells containing nuclear inclusions might appear. Consequently, two young guinea pigs were injected with emulsions made from the pancreas of an old guinea pig and seven young guinea pigs were inoculated with emulsions made from the submaxillary glands of full grown rabbits. No symptoms resulted from any of these inoculations and the microscopic examination of the brains failed to reveal any lesions. Moreover, nine young rabbits, inoculated intracerebrally with an emulsion made from the submaxillary glands of full grown rabbits, remained well and no lesions were found in the brain. The results so far reported, therefore, indicate that an infectious agent is probably responsible for the lesions found in the submaxillary glands of full grown guinea pigs and that the lesions in young guinea pigs resulting from the inoculation of the emulsion of submaxillary glands of full grown guinea pigs are due to this infectious agent.

Transmission in Series.—This conclusion would only be justifiable, however, if it were possible to reproduce the lesion after passage through a series of animals. But in spite of the fact that young guinea pigs which have received intracerebral inoculations of emulsion of submaxillary glands of old guinea pigs almost invariably show severe symptoms with marked cerebral lesions, the inoculation of emulsions of the brains of these experimentally infected animals into other young guinea pigs has invariably failed to cause symptoms or to give rise to lesions. But even though the second animal has shown no lesions or symptoms the inoculations have been continued from animal to animal in series through as many as six guinea pigs with the hope that in this way the virus might gradually acquire greater virulence. But the results have all been negative. Attempts were also made to transmit the virus by inoculating from testicle to testicle or from salivary gland to salivary gland. Except in one instance, to be mentioned later under Series C, when a testicle to testicle inoculation gave a mildly successful result, these experiments were also unsuccessful. Recourse was then had to the expedient of varying

the seat of inoculation, inoculating first in the brain and then in the testicle or *vice versa*. A large number of these experiments have been carried out and in seven instances has it been possible to obtain a positive result on the second transfer. In three of these experiments the transfer was made from brain to testicle, in three others from testicle to brain, and in one from testicle to testicle. In one series it has been possible to reproduce the lesions through a series of three transfers (Series T). In this instance the transfer was made through testicle, brain, testicle. The following are abstracts of the protocols of the experiments in which positive results were obtained.

Series M—Brain to Testicle.—March 1, 1926, Guinea Pig 1 received an injection into the brain of 0.1 cc. of an emulsion of the submaxillary glands of two full grown guinea pigs. In this animal the temperature was elevated from the 3rd to 7th days following the injection, reaching 105.8°. The guinea pig showed marked symptoms, became prostrated, and was killed on the 8th day. Sections from the brain of this animal showed a marked meningeal exudate, and typical nuclear inclusions were found in many of the cells. The remainder of the brain was emulsified and 0.1 cc. of the emulsion was injected into each of the testicles of Guinea Pig 2. In this animal the temperature was elevated to 104.4–105° from the 3rd to the 11th days. It was killed on the 11th day. Sections from the left testicle showed a mononuclear infiltration and a few cells contained typical nuclear inclusions.

Series N—Brain to Testicle.—March 26, 1926, Guinea Pig 3 received an intracerebral injection of 0.1 cc. of an emulsion made from the submaxillary glands of two full grown guinea pigs. The animal became very sick on the 3rd day and was killed. Sections from the brain showed a marked meningeal infiltration with numerous cells showing typical nuclear inclusions. The remainder of the brain was emulsified and 0.1 cc. of the emulsion was inoculated into each of the testicles of Guinea Pig 4. The animal showed an elevation of temperature from the 7th to the 9th days, ranging from 104.4–105.2° and was killed on the 9th day. Sections from the testicle showed a slight cellular reaction and in a few cells typical nuclear inclusions were found.

Series P—Brain to Testicle.—March 18, 1926, Guinea Pig 5 received an intracerebral injection of 0.1 cc. of an emulsion of the submaxillary glands of two full grown guinea pigs. The animal showed an elevation of temperature and marked symptoms, and died on the 4th day. An emulsion made from the brain of this animal was injected into both testicles of two young guinea pigs, Nos. 6 and 7. On the 7th day Guinea Pig 7 showed a temperature of 105.4° and was killed. In sections of the testicles, however, no lesions were found. Guinea Pig 6 had a temperature ranging from 104.9–105.6° on the 7th to 9th days. The animal was killed on the 9th day and in sections of the testicle a circumscribed mononuclear

reaction was seen and in the cells of the tubules in this area there was found a small number of cells, in the nuclei of which were typical acidophilic masses.

Series C—Testicle-Brain: Testicle-Testicle.—January 5, 1926, Guinea Pig 8 received an injection into the left testicle of 0.1 cc. of an emulsion made from the submaxillary gland of an adult guinea pig. This animal had an elevated temperature from 104.8–105.7° on the 6th, 7th, and 8th days, and it was killed on the 8th. Sections made from the testicle showed a marked infiltration with mononuclear cells, and in a small number of the tubule cells, typical nuclear inclusions were found. The remaining portion of the testicle was emulsified in Locke's solution and 0.1 cc. was injected into the brain of Guinea Pig 9 and into the left testicle of Guinea Pig 10. Guinea Pig 9 showed an elevation of temperature on the 5th to the 9th days, ranging from 104.7–105.8°. On the 9th day this animal was killed and sections made from the brain showed a circumscribed meningitic exudate in which were numerous cells showing typical changes with nuclear inclusion bodies. The testicle of Guinea Pig 10 was removed on the 9th day. Sections from this showed a very slight interstitial reaction, a few of the cells of which showed typical nuclear inclusions.

Series T—Testicle-Brain-Testicle.—April 15, 1926, Guinea Pig 11 received an inoculation into the left testicle of an emulsion made from the submaxillary glands of two full grown guinea pigs. The temperature was elevated on the 6th and 7th days, rising to 106° on the 8th day. On this day the animal was killed, the testicle removed, and placed in 50 per cent glycerol. On the following day the testicle was washed free of glycerol and emulsified in Locke's solution. 0.1 cc. of this emulsion was injected into the brain of each of two guinea pigs, Nos. 12 and 13. Beginning with the 3rd day following the injections, both animals showed an elevation of temperature, ranging between 104° and 106°. On the 7th day Guinea Pig 12 was killed. A small piece of the brain was retained for microscopic study and the remainder was placed in 50 per cent glycerol. On the 9th day following the injection, Guinea Pig 13 was killed, the brain removed, a small piece placed in Zenker's fluid for microscopic study, and the remainder placed in 50 per cent glycerol. The examination of the sections of the brains of Guinea Pigs 12 and 13 revealed, in each case, a localized meningeal reaction, and a moderate number of the cells showed characteristic changes with the nuclei containing acidophilic masses.

The brains of Guinea Pigs 12 and 13 were preserved in glycerol for 25 and 27 days, respectively. An emulsion was made from this glycerolated material on May 28 and 0.3 cc. of this was inoculated into the brain of Guinea Pig 14 and 0.1 cc. into each testicle of Guinea Pig 15. Although Guinea Pig 14 had some fever there were no marked symptoms and it was killed on the 11th day following the injection. Sections made from the brain of this animal showed no lesions of any kind.

Guinea Pig 15 had moderate fever up to 105°. It was killed on the 11th day, and the testicles were removed. The left testicle was preserved for further inoculation and the right placed in Zenker's solution. Sections made from this testicle

showed a slight cellular infiltration and edema and in the tubules a small number of cells with nuclear inclusions were found.

It will be seen from these protocols that in seven instances it has been possible to produce lesions in two animals in series and in one other instance in three animals in series. Many variations in the technique have been made in the hope of transmitting the virus indefinitely. Transfers were made at various periods following the infection, even as early as the 2nd day. In other experiments, instead of employing the entire brain tissue for the emulsion, only scrapings from the surface of the brain were used, since the lesions containing the cells with nuclear inclusions are found only in the meningeal exudate. In other experiments it was thought possible that some stimulating or accessory substance present in the submaxillary gland might be necessary for infection and that when transfers were made from brain to brain this factor would of course be lacking. Consequently, emulsions of submaxillary glands of very young pigs were added to the brain emulsions of infected guinea pigs which were used for transfer. The results with none of these methods, however, proved successful.

Although it was not possible to transmit the virus indefinitely, the results obtained offer considerable evidence that in this condition we are dealing with an agent which reproduces itself, and, therefore, presumably is a living virus. There has been no indication so far obtained that the virus on passage tends to become more virulent. Indeed, the opposite effect has been observed. In all cases when any effect has resulted from the second transfer the lesions have been less well marked than those following the first transfer. Although the virus may be preserved, at least for short periods, in glycerol, no evidence was obtained that any increase in virulence or infectivity occurs during this time.

Infectiousness for Other Species of Animals.—Attempts have been made to reproduce the lesions in other species by the inoculation of an emulsion of the submaxillary glands of full grown guinea pigs into the brains of nine young rabbits, five young rats, and two young kittens. These animals all remained well and sections of the brains of these animals, which were killed at varying intervals, showed no lesions.

Properties of the Virus.

Thermolability of the Infectious Agent.—On February 3, a full grown guinea pig was killed and the submaxillary glands removed with sterile precautions. Histologic sections prepared from small pieces of these glands were subsequently shown to contain the specific lesions. Aerobic and anaerobic cultures of the glands made at this time remained sterile. The glands were ground thoroughly in a mortar and suspended in 5 cc. of Locke's solution. After centrifuging for a few minutes at low speed the suspension was divided into two parts; one half was heated at 54°C. for 1 hour and the other was stored on ice during this period. 0.1 cc. of the unheated suspension was then injected intracerebrally into Guinea Pigs 16, 17, and 18, and 0.1 cc. of the heated suspension was injected intracerebrally into each of the guinea pigs, Nos. 19, 20, and 21. All the pigs were less than 1 month old. One of the animals inoculated with the heated material, Guinea Pig 20, was found dead on the 2nd day after inoculation. The brain was removed and prepared for histologic examination. One of the animals which had been inoculated with the unheated material, Guinea Pig 18, was killed on the same day and sections were prepared from the brain for comparison with the sections from Guinea Pig 20.

On February 6, a marked contrast was noted between Guinea Pigs 16 and 17, inoculated with the unheated suspension, and Guinea Pigs 19 and 21, inoculated with the heated suspension. The former appeared unsteady on their feet and their hair was ruffled, whereas Guinea Pigs 19 and 21 appeared perfectly normal. All four guinea pigs were killed on the 3rd day following injection, and histologic sections were prepared from the brains. Microscopic examination of the brains of Guinea Pigs 16, 17, and 18 all showed a marked mononuclear exudate into the meninges in which numerous cells with characteristic nuclear inclusion bodies were found. On the other hand, sections from the brains of Guinea Pigs 19, 20, and 21 showed no meningeal exudate and no cells containing nuclear inclusions.

Resistance of the Virus to 50 Per Cent Glycerol.—On May 17 small pieces of the submaxillary gland of three full grown guinea pigs were placed in a small sterile bottle containing equal parts of glycerol and Locke's solution. The bottle was then stored on ice. A small piece of each gland was prepared for histologic examination and was subsequently shown to contain the specific lesion. The remainder of the glands was emulsified in the usual manner and 0.1 cc. was injected intracerebrally into each of two young guinea pigs, Nos. 22 and 23. Guinea Pig 22 was moribund on the 5th day following injection and was killed. Guinea Pig 23 was found dead on the 7th day. Microscopic study of the brains of Guinea Pigs 22 and 23 showed the usual brain lesion with typical nuclear inclusion bodies. On May 28, 11 days after placing the submaxillary glands in 50 per cent glycerol, the pieces of tissue were washed free of glycerol, ground in a mortar, and suspended in Locke's solution. After centrifuging a few minutes at low speed, 0.1 cc. was injected intracerebrally into each of the guinea pigs, Nos. 24 and 25, both less than 1 month old. Guinea Pig 25 was found dead on the 7th day following the

injection and Guinea Pig 24 was found dead on the 10th day. Microscopic study of the brains of both of these animals showed a meningeal exudate containing cells which showed typical nuclear inclusion bodies.

In another experiment in which the submaxillary gland was exposed to 50 per cent glycerol for 7 days, a similar result to that described above was obtained. In another instance the submaxillary gland was exposed to 50 per cent glycerol for 28 days. The injection of this material into the brains of young guinea pigs failed to produce the characteristic cerebral lesions.

Filterability of the Virus.—On April 15 two full grown guinea pigs were killed and the submaxillary glands removed with sterile precautions. Sections prepared from small pieces of these glands were subsequently shown to contain the specific lesion in the ducts of the glands. Aerobic and anaerobic cultures of the glands made at this time remained sterile. The glands were ground thoroughly in a mortar and suspended in a total volume of 15 cc. of Locke's solution. The suspension was centrifuged at moderate speed for 15 minutes. Half of the supernatant fluid was then filtered through a new Berkefeld N filter. The material filtered rapidly. The filter was subsequently tested and found to be impermeable to *B. coli*. 0.1 cc. of the unfiltered suspension was inoculated intracerebrally into each of three guinea pigs, Nos. 26, 27, and 28. 0.15 cc. of the filtered material was inoculated intracerebrally into each of three guinea pigs, Nos. 29, 30, and 31. All the guinea pigs were less than 1 month old. The results following injection of the unfiltered material were as follows: On April 19, the 4th day following injection, Guinea Pig 26 was found dead, No. 27 was moribund, and No. 28 seemed sick and was killed and the brain removed for histologic examination. Microscopic study of the brain of No. 28 showed an intense meningitis containing numerous cells showing typical nuclear inclusion bodies.

The results following the injections of the filtered material were as follows: Guinea Pigs 29, 30, and 31 all showed on the 2nd day, a rise in temperature ranging from 105–105.6°. On the 4th day Guinea Pig 29 had a temperature of 105.2°. On the 5th day the temperature began to drop and on the 6th day it was subnormal and the animal was killed. The brain was removed and prepared for histologic examination. Guinea Pig 31 showed a rise in temperature, ranging from 104–105° from the 4th to the 8th days. The animal was killed on the 8th day and the brain was removed for histologic examination. The temperature of Guinea Pig 30 ran an irregular course. This animal was killed on the 12th day and the brain removed. A microscopic study of the brains of these three guinea pigs showed a moderate meningitis and in every instance cells containing typical nuclear inclusion bodies were found.

In a second filtration experiment a new Berkefeld N filter was used which was tested during the course of the filtration by the addition of 0.5 cc. of an 18 hour broth culture of *B. coli* to the suspension of submaxillary gland. Cultures of the filtrate remained sterile. The same result as the one described above, was obtained.

Relation of the Infection of the Submaxillary Glands to Other Diseases of Guinea Pigs.

The only other disease affecting guinea pigs known to the writers which may possibly be related to the infectious process in the submaxillary glands is a condition described in 1911 by Römer (12). He observed sporadic cases of paralysis of the extremities in guinea pigs. By intracerebral inoculation of healthy guinea pigs with emulsions of the brains of the diseased animals he was able to transmit the infection without difficulty. After an incubation period of from 9 to 23 days the inoculated animals developed paralysis and after 2 to 10 days of severe illness, died. The brains of both the spontaneously and experimentally infected animals showed a marked infiltration of the meninges with an exudate containing many mononuclear cells and also many polymorphonuclear cells. No mention was made of the presence of cells containing nuclear inclusions.

The brain lesions in this condition resemble to some extent the lesions observed after intracerebral injection of the virus from the submaxillary glands. However, the ease with which the infection could be indefinitely transmitted and the long incubation period observed render it unlikely that the agents concerned in the two conditions are identical, though this possibility should be borne in mind.

SUMMARY AND CONCLUSIONS.

In the lesions of herpes simplex and similar conditions due to filterable viruses, cells are present which show characteristic alterations, particularly in the nucleus. The nucleus of these cells contains a mass which stains with acid dyes. Surrounding this mass is a clear space or halo, within which there are large granules staining with basic stains. These cells are little if at all enlarged.

In a few human cases, especially in infants, enlarged cells have been found which contain nuclei showing changes similar to those seen in the abnormal cells of herpes simplex.

In the ducts of the submaxillary glands of guinea pigs, Jackson observed structures which she considered to be protozoan parasites. Our own studies indicate, however, that these structures are greatly swollen epithelial cells with nuclei having the same characters as the

nuclei of the atypical cells in the lesions of herpes simplex. These cells are usually surrounded by a mononuclear cellular reaction. They were found in 84 per cent of the full grown guinea pigs examined but they were present in only three of forty-three young guinea pigs less than 1 month old. The resemblance of these cells, except as regards size, to the atypical cells present in lesions due to filterable viruses suggested that they also may be the result of an infection with a similar agent. That they are usually not present in guinea pigs less than 1 month old indicates that natural infection usually occurs after this period.

Experiments were therefore undertaken to determine whether or not an infective agent is concerned in this condition and if so to learn something of its nature. When an emulsion of the submaxillary glands of full grown guinea pigs is injected into the brains of young guinea pigs the animals have fever and exhibit symptoms of cerebral irritation. They usually die in 5 to 7 days and in sections of the brain a diffuse subacute meningitis is found. In the exudate there are large numbers of cells having all the characteristics of the abnormal cells of herpes simplex. Similar cells are present in the lesions resulting from the injection of the same emulsion into the testicle, lung, tongue, and submaxillary glands of young guinea pigs. In none of these lesions, however, are the cells greatly enlarged as they are in the lesions in old guinea pigs.

These results support the view that the lesion in the submaxillary gland of old guinea pigs is due to an infective agent. Attempts were therefore made to transmit this agent through a series of young guinea pigs. When the injections were all made into the same organ all the experiments but one gave negative results, but when the site of injection was changed at each transfer it was possible in a number of instances to reproduce the lesions through two animals in series and in one experiment through three animals in series. By modifying the technique, efforts were made to transmit the infection indefinitely but these attempts were unsuccessful. No explanation can be offered for this failure.

Studies made to determine some of the properties of the infective agent have shown that it is destroyed by heating at 54° for 1 hour, and that it is not injured by preservation in 50 per cent glycerol for as long as 11 days. After the material had remained in 50 per cent glycerol

for 28 days, however, it was found to be no longer infective. The infective agent was not held back by a Berkefeld N filter which was impermeable to bacteria. It seems probable therefore that the infective agent belongs in the group of filterable viruses, though further work will be necessary to learn more of its exact nature. These observations present additional evidence that the presence of cells with nuclear inclusions in any lesion indicates that the injury is probably due to an infective agent belonging in the group of filterable viruses.

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EXPLANATION OF PLATE 33.

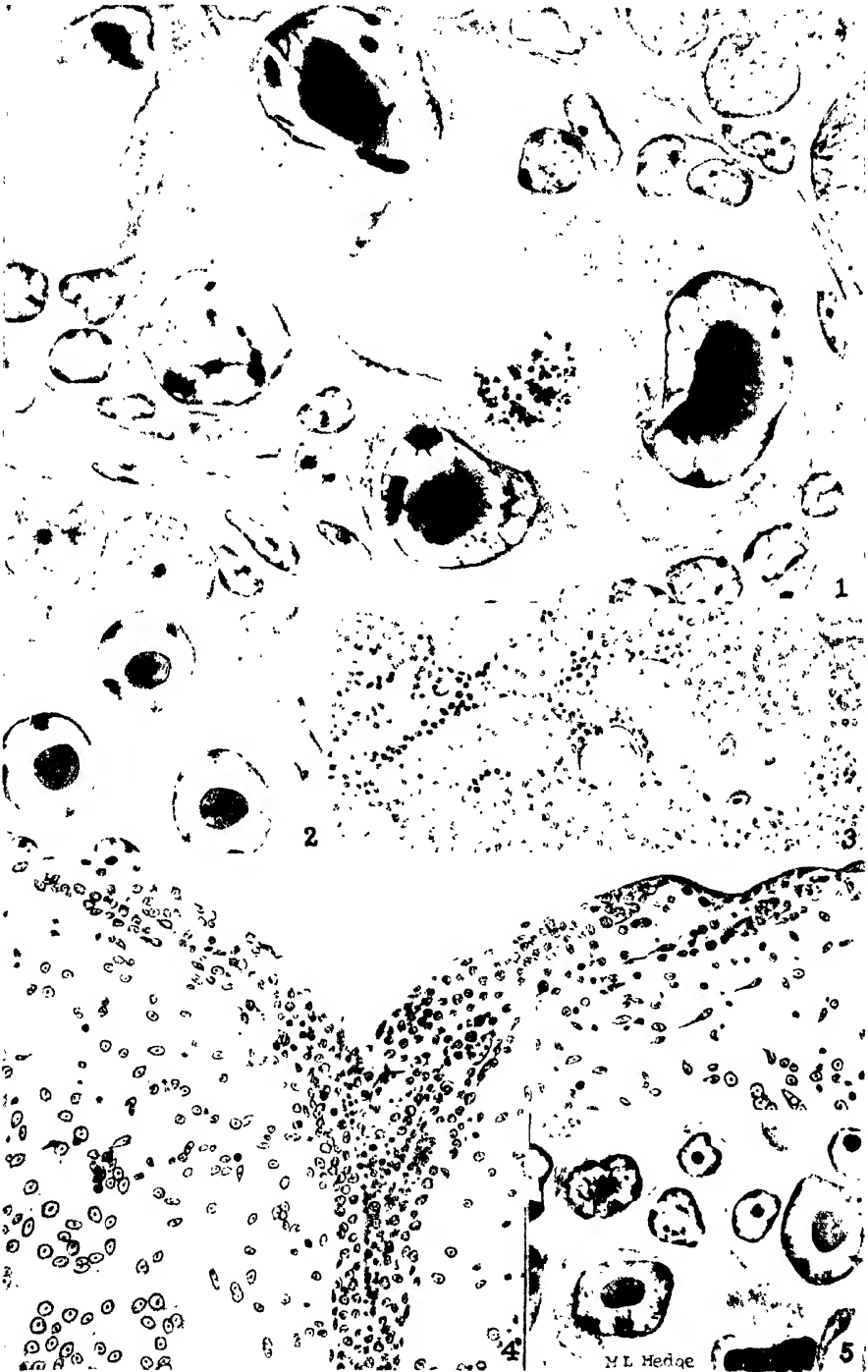
FIG. 1. Swollen epithelial cells containing nuclear acidophilic inclusions within a duct of the submaxillary gland of a full grown guinea pig. Magnification $\times 1700$.

FIG. 2. Duct cells of smaller size containing acidophilic nuclear inclusions. This illustration was made from a section of the submaxillary gland of a young pig, exact age not known. Duct cells of this size containing inclusions have been found only rarely. Possibly these cells are in process of transformation. Magnification $\times 1700$.

FIG. 3. Low power drawing from a section made from the submaxillary gland of a full grown guinea pig. Shows a duct swollen with epithelial cells containing nuclear inclusion bodies. There is a moderate degree of cellular reaction in the vicinity of the infected duct.

FIG. 4. Low power drawing of a section of the brain of a young guinea pig inoculated with an emulsion of the submaxillary gland of a full grown guinea pig. A well marked meningeal exudate is shown.

FIG. 5. High power ($\times 1700$) drawing of the cellular meningeal exudate seen in low magnification in Fig. 4.



(Cole and Kuttner: Filterable virus in guinea pigs)

STUDIES CONCERNING THE RELATIONSHIP BETWEEN PNEUMOCOCCI AND STREPTOCOCCI.

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From time to time evidence has been presented that a close relationship exists between *Pneumococcus* and *Streptococcus*. Kruse and Pansini (1), observing the variability of *Pneumococcus* when grown under different conditions, drew attention to the resemblance between certain races of *Pneumococcus* and *Streptococcus* and even suggested the possibility that these bacteria might have their origin in a common, probably saprophytic form of streptococci. Since then a number of observers have brought forth evidence indicating that *Streptococcus hæmolyticus*, *Streptococcus viridans*, *Streptococcus mucosus* and *Pneumococcus* are so closely related that under certain conditions one may change into the other. The evidence for this point of view has recently been greatly supported by the series of publications of Morgenroth and his collaborators and assistants.

Morgenroth, Schnitzer and Berger (2) have reported that by special methods it was possible to transform with great regularity pneumococci into streptococci.

The methods employed were the following: Pneumococci were cultivated in medium containing dead yeast cells or animal charcoal which had absorbed optochin. Under these conditions the pneumococci underwent modifications and were transformed into cocci which possessed properties held to be characteristic for *Streptococcus viridans*. The organisms were now insoluble in solutions of sodium taurocholate, were avirulent for mice, were resistant to the action of optochin and were more sensitive to the action of rivanol than was the original strain. According to these writers, this transformation of pneumococci into streptococci did not occur suddenly but in undergoing the transformation the bacteria passed through several stages or modifications.

Bacteria in the first stage of the process were described as Modification A. While the bacteria in this stage retained most of the characteristic properties of pneumococci, they had become more resistant to the action of optochin. When blood agar plates were made from the Modification A culture, after growing in optochin, it was found that the colonies no longer resembled those of the original culture but now resembled the colonies of *Streptococcus viridans*. Microscopically the organisms appeared as long chains of round individuals; they were bile-insoluble and were only killed by very high concentrations of optochin. This form was called Modification B and was considered to be identical with *Streptococcus viridans*. Upon further growth in artificial medium, or in the animal, and occasionally upon growth in optochin-containing medium, the third form, Modification C, was found to arise. These organisms corresponded in their properties to *Streptococcus hæmolyticus*, that is, they grew on blood agar with more or less hemolysis, were bile-insoluble and optochin-fast. When injected into the subcutaneous tissue of the abdomen of the mouse phlegmons formed. Moreover, these organisms showed the sensitiveness to the action of rivanol which is characteristic for *Streptococcus hæmolyticus*. Occasionally the transition of Modification A into Modification C occurred without passing through the intermediate stage of Modification B.

These observations concerning the changes undergone by pneumococci and streptococci are of great importance not only in their bearing on the classification of these microorganisms, but also in their relation to the pathogenesis of human infection. It has seemed of importance, therefore, to repeat those experiments and to attempt to interpret them in the light of studies made by the writer (3, 4) and others concerning the variations in the characteristics of Pneumococcus which occur under a number of conditions.

It has been found by a number of observers that when pneumococci are grown in various unusual ways, such as in immune serum or in bile, atypical colonies may appear. These have been called R, or rough, colonies in contrast with the S, or smooth, colonies which are the typical pneumococcus colonies. The bacteria of the R colonies are avirulent for mice, have no capsule, do not produce the so called pneumococcus soluble substance, are somewhat resistant to the action of bile and have lost their type specificity. They have been found to retain their properties on further cultivation and even after repeated passage through mice. Blake and Trask (5) have described a third type of colony, probably made up of intermediate forms, the bacteria of which may revert to the typical virulent pneumococci on passage

through mice. The R bacteria although to some degree resembling streptococci of the *viridans* variety have nevertheless been considered by the writer and by most of the workers on this problem to be modified pneumococci.

Methods.

In studying the modifications undergone by pneumococci when exposed to the action of optochin or optochin absorbed on yeast or on animal charcoal, the following methods were employed.

A strain of *Pneumococcus* derived from a single cell of a Type I stock culture was employed. 0.05 cc. of a 1-10 dilution of an 18 hour broth culture of this organism was inoculated into serum bouillon containing varying concentrations of optochin hydrochloride (ethyl hydrocupreine hydrochloride, Zimmer and Co.) or into serum broth containing varying concentrations of optochin absorbed on yeast. These cultures were incubated from 18 to 20 hours and plated on blood agar to detect colony variations. Yeast-absorbed optochin was prepared as follows: A 10 per cent suspension of dried yeast (Fleischmann) in distilled water was made and this was sterilized by heat and then centrifuged. The supernatant fluid was replaced with a 1-10,000 solution of optochin hydrochloride in distilled water and the mixture incubated $\frac{1}{2}$ hour. The suspension was again centrifuged, the supernatant fluid discarded and replaced by twice the amount of 10 per cent serum broth.

For testing bile solubility, 0.5 cc. of sterile, undiluted ox bile was added to 0.5 cc. of a broth suspension of bacteria and incubated for 2 hours at 37°C.

Experimental.

An 18 hour broth culture of *Pneumococcus* was inoculated into broth containing a suspension of yeast-absorbed optochin as described above and incubated 20 hours. After plating on blood agar only one kind of colony appeared and this resembled exactly the S form of colony which has been described in a previous paper (3). The original culture was now passed two more times through the yeast-absorbed optochin medium. From this third culture, plates were made and now a number of small, thin, rough surfaced colonies, like those which have previously been described as R forms, appeared. A study was next made of the changes undergone by pneumococci when grown in broth containing various amounts of optochin without any yeast being present, and also when grown in suspensions of yeast containing no optochin. The culture was grown repeatedly in serum broth containing optochin hydrochloride in concentration of 1-100,000. At the ninth transfer the concentration was increased to 1-50,000 and at the thirtieth transfer, to 1-20,000. Upon plating the culture at intervals during the repeated transfers, no small R colonies appeared. This last experiment was repeated, substituting plain broth for serum broth. Three transfers were made in this medium con-

taining optochin hydrochloride in a concentration of 1-600,000. When this third culture was plated on blood agar, three kinds of colonies were found, S colonies, R colonies and colonies of an intermediate form like those described by Blake and Trask.

The culture was also grown repeatedly in a 10 per cent suspension of heat-killed yeast in serum broth, no optochin being present. Transfers were made at 6 hour intervals on account of the tendency of the organisms to die in this medium. After the fourth transfer in this medium, plating on blood agar showed a great preponderance of colonies of the R form over those of the S forms.

These experiments, which have been repeated a number of times, indicate that in our hands the result of growing typical pneumococci in media containing yeast which has absorbed optochin, or even in media containing optochin alone or yeast alone, is the appearance of modified pneumococci which form colonies identical with those described by a number of writers as R colonies. From the description of the colonies of Modification B as given by Morgenroth and his associates, it seems probable that the bacteria of Modification B are identical with those we and others have described as pneumococci of the R colonies.

Morgenroth and his collaborators consider these to be true streptococci of the *viridans* type. We have called them modified pneumococci. It is true, as Morgenroth and his collaborators state, that these colonies resemble those of *Streptococcus viridans*. Moreover, the bacteria from these colonies have little virulence for mice and they are resistant to optochin and do not dissolve as readily as do typical pneumococci in solutions of bile. The writers we have mentioned state that these organisms are insoluble in solutions of sodium taurocholate and they lay stress on this property as indicating their identity with the bacteria of the *Streptococcus viridans* group. In carrying out the bile test, however, Morgenroth and his associates have employed the method of Levy (6), adding a 2.5 per cent solution of sodium taurocholate to the broth culture. We have modified this technic, employing not only a 2.5 per cent solution of this substance, but also solutions of a higher concentration. The effect of ox bile itself on these organisms has also been tested. Many tests of R cultures have been made, and at the same time tests have been made of twelve different cultures from various sources which had previously been identified by another worker as cultures of *Streptococcus viridans*.

The latter cultures were all found to be insoluble in ox bile and also in sodium taurocholate solutions, even when this was highly concentrated. On the other hand, when the bacteria of the R colonies or what Morgenroth and his associates have called Modification B bacteria, were tested, it has been found that although they are resistant to the action of sodium taurocholate in 2.5 per cent solution, they are dissolved if higher concentrations of this salt be employed. Moreover, if the bacteria be suspended in salt solution instead of in broth, solution occurs even in 2.5 per cent solutions of sodium taurocholate. Finally, if ox bile be employed, these organisms all dissolve, although more slowly than usually occurs with typical pneumococci. Our experiments, therefore, indicate that these modified bacteria are not, properly speaking, bile-insoluble; they possess increased resistance to the action of bile. They, therefore, differ in this respect from bacteria of the *Streptococcus viridans* group which are truly bile-insoluble. They also differ from *Streptococcus viridans* in certain other properties. We have previously noted the readiness with which pneumococci undergo autolysis when they are suspended in saline solutions and kept at 37°C. This readiness to undergo autolysis is seen in pneumococci of all types and is especially well marked in bacteria from the R colonies. On the other hand, streptococci of all varieties undergo autolysis very slowly.

Saline suspensions of six strains each of the S and R forms of pneumococci and of ten strains of typical *Streptococcus viridans* were made and placed in the incubator at 37°C. for 48 hours. At the end of this time the tubes were centrifuged and stained films were prepared from the sediment of each tube. It was found that well formed, Gram-positive cocci were still present in all the tubes containing streptococci, while in the tubes containing the S and R forms of pneumococci no intact cells remained.

We have reported elsewhere (7) that freezing and thawing easily causes disruption of the cell bodies of pneumococci, while this treatment has no effect on streptococci. Pneumococci of the R form obtained by growth in serum and in bile and also modified pneumococci obtained by growth in media containing optochin absorbed on yeast have been tested by these methods. In every instance all these

bacteria have been found to be readily disintegrated by the action of freezing and thawing.

Finally, the immunological properties of atypical pneumococci derived from type-specific strains by growth in broth containing yeast-absorbed optochin were compared with the immunological properties of typical streptococci of the *viridans* group.

We have previously reported observations concerning the immunological reactions of the R forms of pneumococci (4). It was there shown that sera prepared with each of these strains agglutinated all other R forms, no matter how obtained or from what type of *Pneumococcus*. These sera also caused precipitation of the protein derived from all kinds of pneumococci. The interpretation placed on these experiments was that the R forms of pneumococci were pneumococci which had lost the property of producing the pneumococcus type-specific carbohydrate substance.

Immune sera were now prepared by injecting rabbits with cultures of *Streptococcus viridans*. Six different strains were employed, an immune serum being produced against each one of these strains. These special strains were selected because they grew diffusely in broth. Each of these sera was found to be specific for the homologous organism, causing agglutination usually in dilutions as high as 1-320. In the concentrated serum there occurred some crossing or non-specific agglutination of the other *Streptococcus viridans* strains. All but one of the sera also agglutinated suspensions of R pneumococci but the agglutinating power for these organisms was not as great as that of the serum prepared by the injection of R pneumococci. By absorption of the agglutinins for the R strains by R organisms the agglutinins of the antistreptococcus sera for heterologous *Streptococcus viridans* strains were removed and the absorbed sera were now race-specific and agglutinated only the homologous race of *Streptococcus viridans*. Anti-R pneumococcus sera, on the other hand, did not agglutinate any of the six strains of *Streptococcus viridans* studied.

The following interpretation of these results, in the light of the work of Heidelberger and Avery (8) seems justifiable. Lancefield (9) has previously shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of *Pneumococcus*. Individual strains of *Streptococcus viridans* also possess a substance

comparable to that of the soluble specific substance of *Pneumococcus* and this endows them with the power of reacting specifically with their homologous serum. To what degree a single specific substance is common to a number of *Streptococcus viridans* strains, only further work will show. The protein of all strains being alike and being common to all pneumococci, the serum produced by each *Streptococcus viridans* strain agglutinates all R pneumococci. The anti-R serum, however, does not agglutinate the *Streptococcus viridans* strain because each of these strains has a specific reacting substance, which masks or prevents the non-specific antiprotein reaction. This is analogous to the immunological phenomena exhibited by the specific pneumococci. These organisms, although they contain the non-specific pneumococcus protein, are not agglutinated by anti-R serum or by anti pneumococcus-protein serum. The explanation offered for the immunological phenomena exhibited by strains of the *Streptococcus viridans* group also receives support from the work of Zinsser on hemolytic streptococci (10).

It seems from all of this work that the atypical forms of bacteria obtained by growing type-specific strains of pneumococci in media containing serum or bile or by growing them in dilutions of optochin, or in yeast-absorbed optochin according to the method of Morgenroth, are not strains of *Streptococcus viridans* but are in fact degraded forms of pneumococci.

The reasons we have adduced for this are as follows:

1. Degraded forms of pneumococci are not bile-insoluble, as Morgenroth and his associates have stated, although they are more resistant to bile than are typical pneumococci.
2. Degraded forms of pneumococci behave like typical pneumococci and not like streptococci in the readiness with which they undergo autolysis and in the ease with which the cell bodies are broken down by freezing and thawing.
3. Immune sera produced by the injection of animals with degraded strains of pneumococci behave toward typical strains of *Streptococcus viridans* just as they do toward specific strains of *Pneumococcus*. Typical strains of *Streptococcus viridans* possess a type-specific immunological mechanism just as do typical pneumococci. The species-specific immunological mechanism possessed by *Streptococcus viridans*

is identical with that of *Pneumococcus*. To this extent *Pneumococcus* and *Streptococcus viridans* are closely related. Whether a typical type-specific strain of *Pneumococcus*, however, is ever changed into one of *Streptococcus viridans* any more than a *Pneumococcus* of Type I is ever changed into one of Type II or of Type III only further work will show. The evidence for this at the present time is not convincing.

Experiments with Hemolytic Streptococci.

Morgenroth and his associates (11) also report that they have been able, by special methods, to convert strains of *Streptococcus hæmolyticus* into those of *Streptococcus viridans*. This change was easily induced by cultivating hemolytic streptococci in serum broth containing rivanol bound to erythrocytes, "haut-pulver," animal charcoal or yeast. Rivanol solutions alone in serum broth were not efficacious. Rivanol (2-ethoxy-6, 9-diaminoacridium hydrochloride) is a salt which is said to have a specific germicidal action for *Streptococcus hæmolyticus*.

Other investigators have also observed hemolytic streptococci change into non-hemolytic forms. Valentine and Krumwiede (12) on one occasion observed the spontaneous appearance of occasional green colonies on a blood agar plate made from a culture of *Streptococcus hæmolyticus* (Strain 32, Dochez, Avery and Lancefield (13)). Bacteria from both kinds of colonies were agglutinable in the original strain antiserum and each absorbed the common antibody. Lehmann (14) obtained green-producing colonies from 3 out of 87 strains of hemolytic streptococci which had been cultivated in milk media.

On account of the bearing which these observations concerning streptococci have on the preceding experiments, it seemed of interest to repeat the experiments of Morgenroth and his associates.

Methods.

A 10 per cent suspension of dry yeast in distilled water was sterilized and centrifuged as described previously. The supernatant fluid was replaced with a 1-1,000 solution of rivanol (Metz) in distilled water and incubated for 1 hour at 37°C. After centrifuging, the sediment was washed three times with distilled water and made up to the original volume. Varying dilutions of this suspension were made in 10 per cent serum broth. 0.05 cc. of a 1-10 dilution of a broth culture of *Streptococcus hæmolyticus* was inoculated into 2 cc. of the respective dilutions of yeast-absorbed rivanol and incubated at 37°C. for 20 hours.

In order to obtain pure line cultures, in certain of the experiments single chains of 3 or 4 cocci were isolated according to the technic described in a previous paper (3). Single cocci are difficult to isolate on account of their small size and resemblance to particulate matter in the agar.

Experimental.

Twenty-six strains of hemolytic streptococci, including stock cultures and freshly isolated strains, were tested. Bacteria were inoculated in serum broth containing rivanol alone and in serum broth containing yeast alone, but in most instances in dilutions of rivanol-yeast suspensions varying in strength from 1-4 to 1-8,000. The organisms tended to die in the stronger concentrations and for prolonged growth required alternate transfers into plain serum broth. Cultures could be transferred indefinitely in dilutions weaker than 1-1,000.

After each transfer the organisms were plated on blood agar, incubated at 37°C. and the plates examined for the presence of atypical colonies. Approximately, 400 transfers were made in the various media and only on the plates made from one of the strains (*Streptococcus* 267) were atypical colonies seen. The variant colonies which occasionally appeared on the blood agar plates among the hemolytic colonies of this strain were anhemolytic, and the organisms derived therefrom were avirulent and bred true on further culture. However, atypical colonies appeared a number of times spontaneously when this culture, without any previous treatment with yeast-absorbed rivanol, was plated on blood agar.

To eliminate the possibility of the variant being a contaminant, 20 single short chains were isolated from the original culture and treated with yeast-absorbed rivanol in dilutions varying from 1-1,000 to 1-8,000. Under these conditions no variant colonies appeared. This experiment does not entirely prove that the variants were contaminants but this explanation seems not improbable.

Two of the original cultures, S3 and S23, were transferred 70 times on plain agar before treatment with yeast-absorbed rivanol. In spite of this preliminary treatment no variants ever appeared.

SUMMARY AND CONCLUSIONS.

Morgenroth and his collaborators grew pneumococci in a medium containing optochin and derived variant forms of bacteria therefrom which were considered to be streptococci of the *viridans* group. We have repeated these experiments and have also derived variant forms. These atypical races, however, we have found to be identical with the R form of pneumococci which have been obtained by various other methods. That these R cultures are still pneumococci and do not belong in the *Streptococcus viridans* group is supported by the following observations:

1. These strains are not bile-insoluble, but are more resistant to the lytic action of this agent than are type-specific pneumococci.

2. R strains behave like pneumococci and not like streptococci in the readiness with which cultures spontaneously autolyze and saline suspensions disintegrate during freezing and thawing.

3. Immunological reactions of the variant pneumococci derived by Morgenroth's method are identical with the immunological reactions of R forms of pneumococci derived by various other means.

The observations of Morgenroth and his associates in regard to the transformation of *Streptococcus hæmolyticus* into *Streptococcus viridans* by treatment with rivanol could not be repeated in this laboratory. No explanation is at hand for our failure to produce the change. It is of course possible that none of the twenty-six strains tested had suitable tendencies to variation or that the technic or reagents employed varied from those of Morgenroth.

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EFFECT OF REPEATED FREEZING ($-185^{\circ}\text{C}.$) AND THAW- ING ON COLON BACILLI, VIRUS III, VACCINE VIRUS, HERPES VIRUS, BACTERIOPHAGE, COM- PLEMENT, AND TRYPSIN.

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The effect of low temperatures on bacteria, viruses, ferments, cells, and the bacteriophage has been observed and reported. The effect of repeated freezing and thawing on some of them has also been studied. The temperatures used, however, as well as the number of freezings and thawings employed, varied greatly, and in many instances the effect on only one or two agents was studied by the same worker. Nevertheless some observers have stated that by means of repeated freezing and thawing it is possible to determine whether certain of these agents are living or dead. In view of the inadequacy of the knowledge concerning this subject it seemed desirable to study further the effect of repeated freezing ($-185^{\circ}\text{C}.$) and thawing on vaccine virus, Virus III, herpes virus, and the bacteriophage as compared with the effect on colon bacilli, complement, and trypsin. The results of the investigation are reported in this paper.

Macfadyen (1-3) exposed vegetative and spore forms of bacteria, seed, and Buchner's zymase to the temperatures of liquid air ($-185^{\circ}\text{C}.$) and liquid hydrogen ($-252^{\circ}\text{C}.$) for 20 hours and found that the bacteria grew, that the seed germinated, and that the zymase was still active. No determinations of the number or amount of the active agents, however, were made before or after the exposures.

White's (4) report concerning the results obtained by exposing bacteria to the temperature of liquid air for a period of 2 hours shows that 50 per cent of the hardier bacteria survived while 98 per cent of the less resistant varieties were killed. He also states that vaccine virus was active after an exposure for 15 minutes to the temperature of liquid air. No determinations of the amount of active virus were made, however, before or after the exposure.

Barratt (5) found that rabies virus was active after an exposure for 3 months

to the temperature of liquid air. The same virus, however, was not active after treatment for 45 minutes in a disintegrating machine kept at a temperature of -185°C .

Salvin-Moore and Barratt (6) exposed cells of a graftable mouse cancer to the temperature of liquid air for 30 minutes and found that mice inoculated with the treated cells developed cancers. The observers assume that the cells were killed by the treatment while the cancer virus survived.

Gaylord (7) found that cells of a transplantable mouse cancer, although injured, were able, after exposure for 80 minutes to the temperature of liquid air, to produce tumors when injected into mice. On the other hand, he found that freezing, by means of liquid air, killed embryonic tissue. He also observed that trypanosomes were able to resist a temperature of -185°C . for a period of 20 minutes but not for a period of 40 minutes.

Stockman and Minett (8) state that the virus of foot and mouth disease is active after repeated freezing and thawing. Ammonia brine was used for freezing.

Sanderson (9), using two strains of bacteriophage, one active for staphylococci, the other lytic for colon bacilli, found that the titer of the phages was not influenced by freezing and thawing 20 successive times. Solid CO_2 , approximately -78°C ., was used for freezing. The results of his experiments lead him to believe that the bacteriophage is something other than a viable organism.

D'Hérelle (10), working with two strains of bacteriophage, one active for dysentery bacilli, the other lytic for staphylococci, found that the phage in filtrates less than 17 days old resisted at least 3 exposures to the temperature of liquid air for periods of 10 minutes each, while the phage in older filtrates was not active after 1 to 3 such exposures.

Methods and Materials.

Freezing and Thawing.—The low temperature (-185°C .) used in the experiments to be reported was obtained by means of commercial liquid air which was transported from the plant to the laboratory in Dewar flasks. Desired amounts of the air were transferred to deep Dewar beakers where small amounts of the substances to be frozen, enclosed in Noguchi tubes, were completely immersed for several minutes. After the substances had been completely frozen they were quickly thawed in tap water (16 – 18°C .). This procedure was repeated as often as desired. Then the treated substances, as well as the controls, were tested and titrated by standard methods.

B. coli.—Colon bacilli, 24 hours old, were washed off agar slants and suspended as desired in water, broth, physiological salt solution, or Locke's solution.

Vaccine Virus.—Testicles, into which vaccine virus had been injected 4 days previously, were removed from the rabbit and ground up with sand. After the addition of 20 cc. of Locke's solution, the emulsion was centrifuged, and the supernatant fluid was used as a stock virus. A fresh stock was made for each experiment.

Virus III.—Virus III (11), indigenous to rabbits, was prepared for each experiment in a manner similar to that described for vaccine virus.

Herpes Virus.—Herpes virus was injected into the brain* of a young rabbit. The animal usually died 5 days later. From the brain, removed immediately after death, a 1-10 emulsion was made in Locke's solution and centrifuged. The supernatant fluid was used as a stock virus. Fresh stock was made for each experiment.

Bacteriophage.—A bacteriophage lytic for *B. coli* was prepared in the usual manner in buffered broth with colon bacilli. The resistant bacteria were removed by filtration and the filtrate was used as a stock phage. The filtrates used in the experiments were 19 to 90 days old and the lytic principle in them was usually active in a dilution 10^{-10} . Titrations for the activity of the phage were made in the usual manner by means of dilution with broth in which young colon bacilli were suspended. After the different dilutions were incubated for 48 hours, the cloudy tubes were heated at 60°C. for 30 minutes, and then 0.5 cc. from each tube was tested for the presence of phage. In this way the last tube in which phage was present could be accurately determined.

Complement.—Complement in fresh guinea serum was used. Dilutions of the serum were made by means of physiological salt solution. Activity of the complement was tested in the usual way by dilution or by the determination of the length of time required by the complement to hemolyse a known amount of red blood cells. Care was taken not to inactivate any of the complement by shaking.

Trypsin.—Trypsin, in a buffered solution of pH 7, was supplied by Dr. Northrop. Determinations of the ferment's activity before and after freezing and thawing were made by Dr. Northrop. Details of the technic can be obtained from his papers (12). Care was taken to inactivate none of the ferment by shaking.

EXPERIMENTAL.

All experiments made to determine the effect of freezing ($-185^{\circ}\text{C}.$) and thawing on colon bacilli, Virus III, vaccine virus, bacteriophage, complement, and trypsin were repeated several times, and many of them were performed simultaneously. For convenience, however, the results of the experiments will be reported separately.

Colon Bacilli.

It is a well established fact that many bacteria are killed by repeated freezing and thawing, even when a temperature only a few degrees below $0^{\circ}\text{C}.$ is employed. Therefore colon bacilli were in-

* All operations were performed under ether anesthesia.

cluded as a control in the list of active agents to be exposed repeatedly to the temperature of liquid air, and the following experiment is illustrative of the results obtained with these organisms.

Colon bacilli, washed from an agar slant and suspended in broth, were frozen and thawed 12 successive times. Before freezing there were 180,000,000 viable organisms per cc. After freezing and thawing, however, only 40,000 organisms per cc. remained viable.

When it had been shown by the experiment above that colon bacilli are killed by repeated exposure to the temperature of liquid air, the following experiment was performed to determine what influence the number of bacteria per cc. and the type of suspending fluid have on the percentage of organisms killed by repeated freezing and thawing.

TABLE I.

Summary of Experiment Showing the Effect of Dilution and Diluent on the Percentage of Colon Bacilli Killed by Freezing and Thawing 4 Successive Times.

Diluent	Dilution	No. bacteria per cc. before freezing	No. bacteria per cc. after 4 freezings and thawings
Locke's solution.....	1-10	18,400,000	27,000
" "	1-1,000	380,000	20
Broth.....	1-10	44,000,000	13,200,000
"	1-1,000	600,000	188,000

Colon bacilli, removed from an agar slant, were suspended in sterile distilled water. 0.5 cc. of the suspension was put in 4.5 cc. of buffered broth and Locke's solution respectively. Further dilutions, 1-1,000, were made with each diluent, and two of the suspension, 1-10 and 1-1,000, in each series were frozen and thawed 4 times. The number of viable organisms per cc. was determined before and after freezing. The results of the experiment are summarized in Table I.

It is obvious from the results shown in Table I that the colon bacilli were more susceptible to repeated freezing and thawing when Locke's solution instead of broth was employed as a suspending fluid. It is also evident that approximately the same percentage of bacteria was killed in the different suspensions made by means of broth. On the other hand, a greater percentage of bacteria was killed in the 1-1,000 dilution made with Locke's solution than in the 1-10 dilution made with the same fluid.

Virus III.

The effect of repeated freezing and thawing on Virus III was determined by means of the following experiments.

1 cc. of a fresh stock emulsion containing active Virus III was added to 9 cc. of Locke's solution. One half of the emulsion was used as a control; the other was frozen and thawed 22 successive times. From the second half samples were removed for tests after the 2nd, 12th, and 22nd freezings. The control emulsion and the samples of the treated one were tested for the presence of active Virus III by means of inoculations in the shaved skin of the same normal rabbit. The results of the tests are shown in Table II.

TABLE II.

Summary of the Results Obtained by Repeated Freezing and Thawing of Virus III.

No. of freezings and thawings	Dilutions		
	1-10	1-100	1-1,000
Control (unfrozen)	++	+±	+
2	++	+	±
12	—	—	—
22	—	—	—

+ indicates the presence of a virus reaction at the sites of inoculation. — indicates the absence of a reaction. Dilutions are in terms of the stock testicular emulsion containing the virus.

The experiment indicates clearly that Virus III is easily killed or inactivated by repeated freezing and thawing in spite of the high protein content of the emulsion containing the virus.

Vaccine Virus.

Vaccine virus in a stock testicular emulsion, diluted 1-10, 1-100, and 1-1,000, showed no appreciable decrease in its titer after 12 successive freezings and thawings. Finally, however, it was possible as will be shown in the following experiment, to decrease the titer of the virus by repeated freezing and thawing.

1 cc. of a stock testicular emulsion containing active vaccine virus was diluted 1-10, 1-100, and 1-1,000 by means of Locke's solution. One half of the 1-1,000 dilution was used as a control; the other half was frozen and thawed 34 successive

times. Samples of the latter half were removed for tests after the 24th and 34th freezings. The control emulsion and the samples of the treated one were tested for the presence of active vaccine virus by means of inoculations in the shaved skin of the same normal rabbit. The results of the tests are summarized in Table III.

From the results of the experiment just described it is evident that a certain percentage of the vaccine virus, diluted 1-1,000 with Locke's solution, was inactive after 34 successive freezings and thawings. This virus, however, proved to be more resistant than any other of the active agents tested.

TABLE III.

Summary of the Results Obtained by Repeated Freezing and Thawing of Vaccine Virus.

No. of freezings and thawings	Dilutions		
	1-1,000	1-10,000	1-100,000
Control (unfrozen)	+++	++	+
24	++	+	±
34	++	+	-

+ 's indicate the presence of a virus reaction at the sites of inoculation. - indicates the absence of a reaction. Dilutions are in terms of the stock testicular emulsion containing the virus.

Herpes Virus.

The activity of herpes virus in a fresh stock emulsion was not appreciably decreased by 12 successive freezings and thawings. The following experiment, however, shows that the virus is susceptible to such treatment under certain conditions.

0.5 cc. of a fresh stock brain emulsion containing active herpes virus was diluted 1-20 by means of Locke's solution. One portion of the diluted emulsion was used as a control; another portion was frozen and thawed 24 times. The two portions were then tested for the presence of active virus by means of intracerebral inoculations in young rabbits. The results of the tests are summarized in Table IV.

The experiment demonstrates that herpes virus, provided the brain emulsion containing it has been sufficiently diluted by means of Locke's solution, is killed or inactivated by repeated freezing and thawing.

Bacteriophage.

Early in the work with a bacteriophage lytic for colon bacilli it became evident that repeated freezing and thawing inactivated at least 99 per cent of the phage. Details of these experiments will be omitted since results similar to the ones obtained in them will be

TABLE IV.

Summary of Results Obtained by Repeated Freezing and Thawing of Herpes Virus.

No. of freezings and thawings	Dilutions		
	1-20	1-40	1-80
Control (unfrozen)	+	+	+
24	-	-	-

+ indicates death of the animal within 7 days after intracerebral inoculation of 0.25 cc. of the emulsion diluted as noted. - indicates that the animal was alive 4 weeks after inoculation. Dilutions are in terms of the stock emulsion.

TABLE V.

Summary of Experiment Showing the Effect That the Type of Diluent Has on the Percentage of Bacteriophage Inactivated by Freezing and Thawing.

Diluent	Dilutions of stock filtrate frozen and thawed	Titer of phage after 12 freezings and thawings
Broth (unfrozen control).....	1-10,000	10^{-11}
"	1-10,000	10^{-7}
Distilled water.....	1-10,000	10^{-8}
Locke's solution.....	1-10,000	10^{-8}
Salt "	1-10,000	Completely inactivated

Dilutions are in terms of the stock filtrate.

shown in other experiments performed to ascertain what influence the degree of dilution or the type of diluent has on the percentage of phage inactivated by repeated freezing and thawing. The effect that the type of diluent has on the percentage of phage inactivated under these conditions was investigated first in the following manner.

Portions of the stock filtrate containing the active lytic substance were diluted 1-10,000 respectively by means of buffered broth, Locke's solution, distilled

water, and physiological salt solution. The pH of the first two diluents was 7.6, while that of the last two was between 7 and 6. Small amounts of the diluted filtrate were frozen and thawed 12 successive times. Then tests were made in the usual way to determine the titer of active phage in the control as well as in each treated specimen. The results of the titrations are shown in Table V.

The experiment affords conclusive evidence (1) that phage is inactivated by repeated freezing and thawing, and (2) that the type

TABLE VI.

Summary of Experiment Showing the Effect That Dilution Has on the Percentage of Bacteriophage Inactivated by Freezing and Thawing.

Diluent	Dilutions of stock filtrate frozen and thawed	Titer of phage before and after 8 freezings and thawings
Broth (control).....	1-10	10^{-11}
" (frozen).....	1-10	10^{-9}
" ".....	1-1,000	10^{-9}
" (control).....	1-1,000	10^{-11}
Distilled water (control).....	1-10	10^{-11}
" " (frozen).....	1-10	10^{-8}
" " ".....	1-1,000	10^{-8}
" " (control).....	1-1,000	10^{-10}
Locke's solution (control).....	1-10	10^{-11}
" " (frozen).....	1-10	10^{-10}
" " ".....	1-1,000	10^{-7}
" " (control).....	1-1,000	10^{-11}
Salt solution (control).....	1-10	10^{-11}
" " (frozen).....	1-10	10^{-9}
" " ".....	1-1,000	10^{-8}
" " (control).....	1-1,000	10^{-11}

Dilutions are in terms of the stock filtrate.

of diluent influences the percentage of phage inactivated in this manner.

After it had been determined that the type of diluent does affect the percentage of phage inactivated by repeated freezing and thawing, the following experiment was performed to ascertain what effect the degree of dilution has on the percentage of phage inactivated by such treatment.

Portions of the stock filtrate containing phage were diluted 1-10 and 1-1,000 by means of each of the following diluents: broth, distilled water, Locke's solution, and physiological salt solution. Half of each specimen was used as a control; the other half was frozen and thawed 8 times. Tests were then made in the usual way to determine the titer of active phage in each specimen. In these titrations pipettes were changed after every third tube. Consequently the titer of the phage in each series may be slightly higher than it would have been had a clean pipette been used after each tube. Since the same technic was used throughout the experiment, the results of the different titrations summarized in Table VI are comparable.

The results of the experiment indicate that an increase in the dilution of the stock filtrate by means of broth and distilled water

TABLE VII.

Summary of Experiment Showing the Effect That Freezing and Thawing Have on Complement in Diluted Serum.

Specimens of complement	Titration by dilution				Time required for complete hemolysis*
	1-50	1-100	1-200	1-400	
1-50 (control)	++	+	±	—	3 min.
1-50 (frozen and thawed).....	+	±	—	—	7 "
1-100 (control).....		+	±	—	4 "
1-100 (frozen and thawed).....		—	—	—	No hemolysis

* Dr. Landsteiner was kind enough to make this test.

+ 's indicate hemolysis. — indicates no hemolysis.

does not at the same time lead to an increase in the percentage of phage inactivated by repeated freezing and thawing. On the other hand, an increase in the dilution accomplished by means of physiological salt solution and Locke's solution does lead to an increase in the percentage of phage inactivated, as evidenced by a greater percentage of phage being inactivated in dilutions of 1-1,000 than in dilutions of 1-10. These findings are similar to the ones shown in Table I concerning the effect of dilution on the percentage of bacteria killed by repeated freezing and thawing.

Complement.

The titer of complement in undiluted serum and in serum diluted 1-10 is not appreciably decreased by 12 successive freezings and

thawings. When this fact became evident, a study concerning the effect of freezing and thawing on complement in highly diluted serum was made in the following manner.

Portions of fresh guinea pig serum were diluted 1-50 and 1-100 by means of physiological salt solution. Half of each specimen was used as a control; the other half was frozen and thawed 12 times. Then tests for the presence of active complement in each of the control and treated specimens were made by means of dilution and also by the determination of the time necessary for given amounts of the sera to completely hemolyse given amounts of red blood cells in the presence of a great excess of amboceptor. Throughout the experiment, the results of which are shown in Table VII, care was taken not to inactivate the complement by shaking.

From the results of the experiment it may be concluded that complement in highly diluted serum is inactivated by repeated freezing and thawing.

Trypsin.

The effect of freezing and thawing on trypsin was studied in the following manner.

Portions of a 5 per cent stock solution of trypsin were diluted 1-10 and 1-500 by means of a buffered solution having a pH of 7. Half of each specimen was used as a control, the other half was frozen and thawed 12 times. Care was taken to inactivate none of the ferment by shaking. After the amounts of active trypsin in the control and treated specimens were determined¹ and compared, it was found that 70 per cent of the trypsin was inactivated in the specimen diluted 1-500, while only 17 per cent was inactivated in the one diluted 1-10.

The results of the experiment above afford conclusive evidence that trypsin is inactivated by repeated freezing and thawing and under these conditions a greater percentage is inactivated in a dilute than in a concentrated solution.

DISCUSSION.

The numerous discussions concerning the nature of the bacteriophage have led many investigators to question more closely the living nature of some of the so called filterable viruses. Many tests have

¹ Dr. Northrop supplied the trypsin and determined the amount of active trypsin in the control and treated specimens.

been devised to act as criteria for the presence of life, but so far no one of them has been found satisfactory. Recently Sanderson (9), using a temperature of $-78^{\circ}\text{C}.$, found no diminution in the titer of two strains of phage subjected to 20 successive freezings and thawings. Since bacteria and cells are killed by repeated freezing and thawing he concludes that the bacteriophage must be something other than a living organism. In the experiments reported in the present paper a number of active agents, some undoubtedly living, others equally unquestionably not living, and still others of a doubtful nature, were subjected to repeated freezing ($-185^{\circ}\text{C}.$) and thawing. By these tests it has been possible to determine that mere destruction or inactivation of a substance cannot be accepted as proof that it existed in a living state.

CONCLUSIONS.

Colon bacilli, Virus III, vaccine virus, herpes virus, bacteriophage, complement, and trypsin are either killed or inactivated by repeated freezing ($-185^{\circ}\text{C}.$) and thawing. As might be expected, some of the agents are more resistant than others.

Hence it may be concluded that destruction or inactivation of an active agent by repeated freezing ($-185^{\circ}\text{C}.$) and thawing is not proof that it was possessed of life.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

V. PRODUCTION OF ANTISEPTICEMOTOXIN BY IMMUNIZATION WITH OXIDIZED PNEUMOCOCCUS HEMOTOXIN.

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INTRODUCTION.

It is known that active or reduced pneumococcus hemotoxin is antigenic since immunization with it induces a so called "antihemotoxin" which neutralizes the lysin (1). There is a marked lessening in the hemolytic activity of oxidized pneumococcus extracts (2), and it has seemed important to determine whether a loss in antigenicity accompanies the inactivation of the hemolytic property. Our investigation of this question has consisted in a comparison of the immunity response to the injection of reduced and oxidized pneumococcus extracts.

EXPERIMENTAL.

Methods.

Substances Injected.—A series of rabbits were immunized by injection of the following substances: (1) "reduced" or active pneumococcus extract which was stored in sealed tubes and subjected to a minimum exposure to air before injection; (2) oxidized or inactive extract which was exposed to air for 6 hours at 37°C. and then stored at 5°C.; (3) "vaccine" or heat-killed suspensions of pneumococci.

The reduced extract contained the active hemotoxin in high concentration, 0.001 cc. producing hemolysis when added to 2.5 cc. of red blood cells. The oxidized extract contained inactive hemotoxin and before each injection was always tested for the presence of traces of active lysin. That no significant amount of it persisted in the oxidized extract was proved by the fact that 0.3 cc. of the extract caused no detectable hemolysis when added to 2.5 cc. of blood cells. The results

in animals injected with the heat-killed bacterial suspension were included for purposes of comparative study. The bacterial suspensions were heated at 56°C. for 30 minutes. Young cultures were used and the suspensions were heated at once to minimize autolysis and cell solution.

Immunization.—The reduced and the oxidized sterile cell solutions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Four courses of injections were given, the doses gradually increasing from 0.1 cc. to a maximum of 2.0 cc., in the last series of injections.

The "vaccine" or heat-killed bacterial suspension was also injected intravenously at the same time as the sterile cell solutions. Four courses of six daily injections of 0.1 cc. of a concentrated suspension of the bacteria were given so that the animals received in all the equivalent of bacteria from 24 cc. of culture.

All of the rabbits were bled on the 10th day after the last injection.

Titration of Antihemotoxin in Immune Serum.—The method used in determining the antihemotoxin value of the immune serum consisted in adding various amounts of serum to a constant amount of hemotoxin and determining the amount of serum required to completely neutralize the lysin. The hemotoxin-serum mixtures were shaken and then incubated for 45 minutes to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated to determine the presence of free or unneutralized hemotoxin. The detailed procedure is given below:

Constant "Dose" of Hemotoxin.—The hemotoxin content of a sterile pneumococcus extract was determined by a preliminary titration. Three times the amount of extract found sufficient to completely hemolyze 2.5 cc. of a 1 per cent suspension of washed rabbit cells was chosen as the test "dose" of hemotoxin. The pneumococcus extract was then diluted to such a concentration that 1.5 cc. of the dilute solution contained this amount of lysin.

Hemotoxin-Serum Mixtures.—0.5 cc. of different dilutions of serum was added to the series of tubes containing 1.5 cc. of the dilute hemotoxin solution. Owing to the inhibitory effect on the lysin of certain constituents of normal serum, control tubes were included containing the sera of the animals from bleedings made before immunization. The mixtures of hemotoxin and serum were shaken and then incubated for 45 minutes at 37°C.

Final Hemolysis Tests.—At the end of the 45 minutes, the presence of free or unneutralized lysin was determined by adding 0.5 cc. of a 5 per cent suspension of rabbit cells to the hemotoxin-serum mixtures. These final hemolysis test mixtures were incubated for 1 hour and readings of hemolysis made after centrifugation.

Control of Lysin "Deterioration" during Incubation of Hemotoxin-Serum Mixtures.—It was necessary to rule out the possibility that the lysin might be inactivated by oxidation since, should this occur, it might be confused with the immunological neutralization. For this purpose, control mixtures of 0.5 cc. salt solution plus 1.5 cc. of dilute hemotoxin were incubated with the series. At the end of the incubation period, these mixtures were diluted one-half and one-third; and the usual amount of red blood cells was added after these dilutions of the

control lysin had been made up to the correct volume of 2.0 cc. The control test mixture containing one-half and one-third the amount of lysin used, always showed complete hemolysis which proves that none of the apparent lysin neutralization or inhibition can have been due to inactivation of hemotoxin by oxidation during the preliminary incubation period.

Absence of Visible Precipitation in the Incubated Hemotoxin-Serum Mixtures.—Titrations of immune serum for the presence of antibodies neutralizing hemotoxins or enzymes are often complicated by the fact that the tested sera contain precipitins for other proteins present in the cell solutions. If during the preliminary incubation of the serum and cell solution, a precipitation or flocculation occurs, it is always possible that the active substance may be carried down with the precipitated particles. Subsequent tests of mixtures in which this precipitation has occurred indicate an apparent inhibition or neutralization of the test substance although in fact the active substance may have been only mechanically removed.

No such source of error existed in our experiments; for there was never any visible clouding or precipitation. The point is important since the immune sera contain a precipitin for the pneumococcus protein present in the bacterial cell solution used to furnish the hemotoxin. When the serum and bacterial solution are less dilute than in mixtures made according to the above procedure, protein precipitation occurs.

Strength of the Antihemotoxin in the Serum of Animals Immunized with Oxidized (Inactive) and Reduced (Active) Pneumococcus Hemotoxin.

In the following experiment a comparison was made of the hemotoxin-neutralizing power of the sera of animals immunized with oxidized (inactive) hemotoxin and that of sera immunized with the reduced (active) hemotoxin. The serum of a rabbit immunized with a heat-killed suspension of intact pneumococci was included for comparison. The general procedure has been described under Methods. In this experiment, the immune sera were tested against hemotoxin derived from pneumococci of the same fixed type (Type II) as the cell solutions used for immunization. The results are given in Table I.

The marked neutralizing power of the sera of animals immunized with both the oxidized (inactive) and the reduced (active) solutions of pneumococci is evident in Table I. The production of an antihemotoxin by the injection of cell solutions containing the active hemotoxin has been shown in Cole's (1) studies. The new fact revealed in Table I is the production of a neutralizing antibody by immunization with oxidized, that is to say, inactive pneumococcus hemotoxin. It is

evident that the serum of the animals injected with an oxidized extract which contains no demonstrable active hemotoxin possesses approximately the same neutralizing action as does that of animals immunized with the reduced, or active, hemotoxin.

The animal immunized with the heat-killed suspension did not produce antihemotoxin. This is an interesting correlation with the similar failure of immunization with properly prepared "vaccines" to yield an antibody for pneumococcus protein which like the hemotoxin is an endocellular substance (4).

TABLE I.

Antihemotoxin in Sera of Animals Immunized with Reduced (Active) Hemotoxin and Oxidized (Inactive) Hemotoxin.

No. of rabbit	Immunized by injection with	Hemolysis by previously incubated mixtures of hemotoxin and serum						
		Immune serum					Normal serum, before immunization	
		Amount of serum					Amount of serum	
		0.01 cc.	0.006 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.05 cc.	0.01 cc.
1	Inactive hemotoxin in oxidized cell solution	0	0	0	0	0	++++	++++
2	" "	0	0	0	+	±	+++	++++
3	Active hemotoxin in reduced cell solution	0	0	0	0	±	+++	++++
4	" "	0	0	0	0	0	+++	++++
5	"Vaccine" (heat-killed suspension of intact cells)	+++	++++	++++	++++	++++	++++	++++

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

In the preceding experiment, the neutralizing capacity of the immune sera was tested against hemotoxin derived from the homologous type of pneumococci. Cole (1) has shown that the antihemotoxin produced by immunization with the active substance is not type-specific,

but is effective against hemotoxin derived from all types of pneumococci. It seemed of interest to determine if the antihemotoxin produced by immunization with inactive (oxidized) hemotoxin is likewise equally effective against the hemotoxin derived from heterologous types of pneumococci. The protocol of an experiment testing this question is presented in Table II.

As shown in Table II the antihemotoxin which is produced by immunization with oxidized or inactive *Pneumococcus* is only species-

TABLE II.

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

Serum of animal immunized against	Hemolysis by previously incubated mixtures of hemotoxin and serum					
	Hemotoxin from Type I <i>Pneumococcus</i>		Hemotoxin from Type II <i>Pneumococcus</i>		Hemotoxin from Type III <i>Pneumococcus</i>	
	Amount of serum		Amount of serum		Amount of serum	
	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.
Inactive hemotoxin in oxidized pneumococcus cell solution (Type II)	0	0	0	0	0	0
Active hemotoxin in reduced pneumococcus cell solutions (Type II)	0	0	0	0	0	0
"Vaccine" (heat-killed suspension of intact cells)	++++	++++	++++	++++	++++	++++

specific and not type-specific since it neutralizes the hemotoxin derived from the cells of heterologous types of pneumococci. A species-specific antibody is apparently the usual type involved in the neutralization of bacterial hemotoxins; for the hemotoxin from cholera vibrios which are type-specific by agglutination, likewise gives rise to a common neutralizing antibody (3). The same holds true of the neutralizing antibody for the true toxins formed by diphtheria, tetanus and Welch bacilli, although each of these bacterial species includes strains which exhibit type specificity in tests with agglutinating immune sera.

Comparison of the Inhibiting Effect of Normal and Immune Serum upon Digitonin Hemolysis.

The serum of normal animals possesses a certain inhibitory action upon pneumococcus hemotoxin. At least a part of this inhibition by normal or non-immune serum can be referred to lipid constituents. In the case of tetanolysin, a number of investigations have proved that the increase in the neutralizing action of immune serum is not due to an increased concentration of the lysin-inhibiting constituents of normal serum, and it might be concluded by analogy that this is also true for the pneumococcus hemotoxin. However, in view of the fact that the antihemotoxin described in the present paper is produced by immunization with a non-hemolytic antigen, it seemed desirable to determine whether the increased neutralizing capacity is due to a non-specific substance which would inhibit the hemolytic activity of other lytic agents.

Digitonin was chosen as the lytic substance to test this question, largely because hemolytically active digitonin is known to combine with cholesterol to form a non-hemolytic compound. This reaction, in fact, is used as the basis of certain methods employed in the quantitative estimation of the cholesterol content of normal serum (4). The combination of cholesterol and digitonin is especially suited to a comparison of the degrees of neutralization of pneumococcus hemotoxin by normal and immune sera since Cole (1) has shown cholesterol to be one of the most important constituents involved in the inhibitory action of normal serum upon the pneumococcus lysin.

The experiments may be outlined as follows: The lytic action of a sample of digitonin was determined in the absence of serum. With this information at hand two dilutions of digitonin were prepared in salt solution; one solution contained in 1.5 cc. twice the amount required to hemolyze 2.5 cc. of a 1 per cent suspension of rabbit cells; the other solution contained four times that amount of the lytic agent. Mixtures of the digitonin with various dilutions of normal and immune rabbit serum were then prepared as described for the test mixture of pneumococcus hemotoxin and serum in the previous experiments. After incubation for 1 hour at 37°C. to allow time for the combination of digitonin with the cholesterol and other serum constituents, 0.5 cc. of a 5 per cent suspension of rabbit cells was added.

The results of these experiments were conclusive. Although relatively large amounts of both the normal and immune serum inhibited the digitonin hemolysis, there was no significant difference in the action of the two. The sera obtained by immunization with both the reduced and oxidized solutions exhibit more than 100 times the neutralizing capacity of normal serum when tested against pneumococcus hemotoxin, yet they exhibited no greater neutralizing or inhibiting action on digitonin hemolysis than did this serum. From the results, it is obvious that the neutralizing antibody produced during immunization with the hemolytically inactive, oxidized hemotoxin is distinct from the usual lysin-inhibiting constituents of normal serum.

Neutralization Experiments with Pneumococcus Immune Serum and Hemotoxins of Other Bacteria.

Experiments were conducted to determine whether the immune sera which neutralize pneumococcus hemotoxin have any neutralizing or inhibiting action upon the hemotoxins of other bacteria. Tetanolysin and the lysin of the Welch bacillus were the hemotoxins tested. Normal serum and the serum produced by immunization with heat-killed pneumococcus vaccine were included as controls, since certain constituents of normal serum have a marked inhibitory action upon the lysins of both of these anaerobic bacilli. The procedure employed was essentially the same as in the previous experiments with the pneumococcus lysin. The constant "dose" of tetanolysin and Welch lysin used in these experiments represents 8 to 10 times the amount of lysin required for the hemolysis of 2.5 cc. of rabbit cells in the absence of serum. A larger "dose" of lysin was chosen than in the previous experiments because these lysins are inhibited to a greater extent by normal serum than is the lysin of pneumococci. The preparation of serum-hemotoxin mixtures and other steps in the procedure were the same previously described.

Three normal sera and the sera of the four rabbits immunized with oxidized and with reduced pneumococcus extracts were included in the experiment. The results of tests upon tetanolysin with a typical normal and a typical immune serum are presented in Table III.

In spite of the markedly increased capacity of the immune serum to

neutralize the hemotoxin of *Pneumococcus*, the results presented in Table III which were typical for the experiments show that immunization of animals with this hemotoxin does not increase the ability of their serum to neutralize or inhibit the hemotoxin of the tetanus bacilli.

The results of the tests with the Welch lysin were not so clean-cut, since they were complicated by marked differences in the neutralizing or inhibiting capacity of different normal rabbit sera—a fact previously observed in another investigation (5). The sera of the various immune rabbits also showed marked differences among themselves in their inhibitory action on the Welch lysin. However, a survey of the results as a whole indicates quite clearly that the sera of rabbits

TABLE III.

Inhibition or Neutralization of Tetanolysin by Pneumococcus Immune Serum and by Normal Serum.

Serum	Hemolysis by previously incubated mixtures of serum and tetanolysin		
	Amount of serum		
	0.005 cc.	0.001 cc.	0.0002 cc.
Rabbit immunized against pneumococcus hemotoxin.....	0.	+	+++
Normal rabbit.....	0	0	++

immunized against the hemotoxin of *Pneumococcus* exerted no greater neutralizing or inhibiting effect upon the lysin of Welch bacilli than was found with the serum of normal or non-immune animals.

COMMENT.

The preceding experiments indicate that a neutralizing antibody may be produced by immunization with the hemolytically inactive hemotoxin present in oxidized solutions as well as by immunization with the active hemotoxin. The antibody is a species-specific antihemotoxin neutralizing the hemotoxin from all types of pneumococci. It seems to be without effect upon the hemotoxins of tetanus and Welch bacilli.

The theoretical and practical importance of "toxoid immunization"

gives a special significance to the above results. But the data thus far procured do not enable one to state that the inactive or oxidized lysin possesses antigenic properties identical with those of the reduced or active hemotoxin. A more complete discussion of the antigenic properties of the inactive oxidation products of pneumococcus hemotoxin will be presented in a subsequent paper.

SUMMARY.

Immunization with solutions of the intracellular substances of pneumococci, in which the hemotoxin has been rendered inactive by oxidation, yields an antibody which neutralizes the active or reduced hemotoxin.

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GASOMETRIC MICRO-KJELDAHL DETERMINATION OF NITROGEN.

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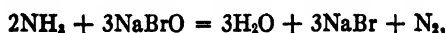
The ease and accuracy with which the manometric blood gas apparatus (4) makes possible the measurement of small amounts of gas evolved from solutions has led us to extend the use of the apparatus to determinations of substances other than the blood gases. The apparatus can be used in any determination in which the final product is a gas, or will enter into a quantitative reaction producing a gas. Thereby the measurement is based, as in gravimetric analysis, on direct observation of the amount of substance obtained, independent of comparison with standard solutions by titration, colorimeter, or otherwise. This advantage of directness and freedom from dependence upon the accuracy of standards is combined with the rapidity and ease of a final measurement by volume.¹

In this paper a method for micro-Kjeldahl determination of nitrogen

¹ Technique with the manometric gas apparatus has been developed for exact urea determinations by measurement of the CO₂ of the ammonium carbonate formed with urease, and for approximate urea determinations for the hypobromite reaction; for calcium determination by precipitation of the calcium as oxalate, resolution in dilute sulfuric acid, and determination of the CO₂ evolved by the practically instantaneous oxidation of oxalic acid with permanganate in the gas apparatus; for amino nitrogen by the nitrous acid reaction; for sugar by permitting it to reduce potassium ferricyanide, and measuring the excess ferricyanide by the N₂ evolved from the reaction with hydrazine. These methods will be reported shortly. We have also attempted to determine phosphoric acid by estimating with hypobromite the ammonia of the magnesium ammonium phosphate precipitate, and, following a friendly suggestion by A. T. Shohl, the ammonia of the yellow ammonium phosphomolybdate precipitate obtained according to Lorenz. The ammonia contents of both precipitates under the conditions thus far tried have, however, been sufficiently variable to cause errors up to as much as ± 7 per cent.

is reported which has proven satisfactory for both experimental work and hospital routine. The ammonia in the Kjeldahl digest is estimated gasometrically by treatment with hypobromite in the manometric apparatus of Van Slyke and Neill. The pressure of 1 mg. of nitrogen measured at 2 cc. volume in the apparatus is about 300 mm., so that accurate readings are easy: duplicate results usually check to within 1 per cent.

The gasometric determination of ammonia by measurement of the nitrogen gas evolved by the reaction with hypobromite,



has been a standard procedure of recognized accuracy for at least 50 years (1). The amount of nitrogen gas evolved falls somewhat short of that calculated by the above equation, but the percentage of the theoretical yield is so constant under defined conditions of hypobromite and alkali concentration that with the introduction of an empirical correction factor Classen states the error may without special precautions be kept within 0.5 per cent of the ammonia determined.

Stehle (2) has used the hypobromite reaction in the original constant pressure blood gas apparatus of the writer to determine the ammonia nitrogen obtained in micro-Kjeldahls. Stehle found (3) that copper used as a catalyst for the sulfuric acid digestion reacted with the hypobromite to cause evolution of appreciable amounts of oxygen gas, which had to be absorbed with pyrogallol before the nitrogen volume could be observed. When this precaution was observed, theoretical results were obtained with sodium sulfanilate, uric acid, and urea, and results consistent with the macro-Kjeldahl for casein and milk. However, the absorption of oxygen was a troublesome additional step in the procedure and Stehle finally (3) abandoned the use of copper catalyzer, and carried out the digestion with only sulfuric acid and potassium sulfate. He found that this procedure gave satisfactory results for non-protein nitrogen determination in blood filtrates.

Our experience confirms Stehle's, that with the blood gas apparatus ammonia determinations by the hypobromite method may be carried out with accuracy. Without catalysts or oxidizing agents to assist in the Kjeldahl digestion, however, the use of the method for micro-

Kjeldahls would be very limited. Low results are obtained with proteins and other substances which offer resistance to digestion. The problem of performing a simple gasometric nitrogen determination therefore resolved itself into finding a satisfactory general catalyzer for the digestion which would not prevent the reaction of the ammonia with hypobromite, nor cause formation of gases other than the nitrogen evolved from the ammonia. Various catalysts and oxidizers previously employed for Kjeldahl determinations were tested. Mercury was found to form a combination with ammonia which resists the action of hypobromite. Perchloric acid if used in excess destroyed part of the ammonia during the digestion. Hydrogen peroxide gave inconstant results. However, when potassium persulfate in great excess as used by Wong (5), was tried, satisfactory results were obtained with regularity. The digestion even of protein material is completed in a few minutes, and with less resistant material such as urine or blood filtrate oxidation is completed so quickly that the dark color of carbon does not even appear. Furthermore, persulfate does not destroy any ammonia. Unnecessary prolongation of the digestion consequently does not injure the results, provided, of course, the overheating is not carried to the extreme of volatilizing ammonium sulfate.

About the only point that requires especial consideration when persulfate is used is the water content of the digest at the time the persulfate is added. If the material to be analyzed is dissolved in several cc. of water per cc. of acid used, and sulfuric acid and persulfate are both added before the water is boiled off, all of the persulfate will decompose during the subsequent boiling down, and before the solution has become concentrated enough for digestion to occur. Results will accordingly be low because of lack of oxidizing agent during the actual digestion. This was pointed out by Wong (5). On the other hand, if the sulfuric acid solution is boiled down till SO_3 fumes come off, and then dry persulfate is added, the latter will not be completely decomposed during the digestion, and will evolve appreciable amounts of oxygen when treated with hypobromite. The mixture of ordinary concentrated sulfuric acid and syrupy phosphoric acid, 3:1, which we finally came to use, appears to have enough moisture in it to act with the persulfate on a dry

substance; and 1 cc. of water is not too much. The procedure adopted for samples dissolved in more than 1 cc. of water was accordingly to boil down with 1 cc. of the acid mixture till fumes began to come off, and to add 1 gm. of persulfate, with 1 cc. of water, to the cooled residue, before finishing the digestion.

The use of phosphoric acid in the digest is not necessary but it accelerates the digestion. 0.1 cc. of blood serum digested with 1 cc. of sulfuric acid and 1 gm. of persulfate requires about 15 minutes to clear. If a mixture of 1 part phosphoric to 3 parts of sulfuric acid is used, instead of sulfuric alone, 5 minutes suffice. A larger proportion of phosphoric acid is likely to attack the glass tubes.

The hypobromite solution used was an object of considerable experiment. Various concentrations of bromine in various concentrations of alkali have been used in the past. All of them form oxygen while standing. Stehle's solution, containing per 100 cc. 2.5 gm. of Br., 2.5 gm. of KBr, and 5.3 gm. of NaOH, was found to form oxygen several times as fast as solutions of bromine in more concentrated NaOH without bromide. The solution finally adopted, 1 cc. of bromine to 50 cc. of 40 per cent sodium hydroxide, generates oxygen so slowly that the amount formed in an hour in 1 cc. of the solution (the amount used for an analysis) exerts at 2 cc. volume only about 0.3 mm. pressure. The presence of 40 gm. of NaOH per 100 cc. reduces the solubility of air in the solution to so nearly zero that it is not necessary to extract the air before the solution is used, the slight trace of air present being included in the blank for the reagents. The amount of bromine present in the 1 cc. of this solution is sufficient to decompose in 1 minute the maximum amount of ammonia for which the method is designed, and it is not enough to cause trouble by the rate of its attack on the mercury in the gas apparatus.

Description of Method.

Reagents.

Sulfuric-Phosphoric Acid Mixture.—3 volumes of concentrated sulfuric acid are mixed with 1 volume of syrupy phosphoric acid (specific gravity 1.7).

Potassium persulfate containing less than 0.01 mg. of ammonia nitrogen per gm.²

40 Per Cent Sodium Hydroxide.—40 gm. of NaOH per 100 cc. of solution, ammonia-free.

Sodium Hypobromite Solution.—To 50 cc. of the 40 per cent NaOH in a 250 cc. flask add under a hood, 1 cc. of bromine, and stir until the bromine is completely dissolved. Gaseous oxygen forms very slowly in this solution. In order to prevent some slight accumulation, however, the hypobromite is aerated by whirling it about the walls of the flask for a few seconds each time before a portion is used for analysis. *The hypobromite solution is made fresh on the same half day in which it is used.*

Procedure.

Digestion.—In a 200 × 25 mm. test-tube of Pyrex glass or silica, place the sample to be analyzed, containing preferably 0.3 to 1.5 mg. of nitrogen, 1 cc. of the sulfuric-phosphoric acid mixture, and a glass bead to prevent bumping. If the volume of water added with the sample is 1 cc. or less, 1 gm. of potassium persulfate is added at once. If a larger volume of water is present, however, it is first boiled down till white fumes appear, the mixture is cooled, and 1 gm. of persulfate is then added, together with 1 cc. of water. Heating is continued until the mixture has become entirely clear, or after white fumes appear, in the case of substances which oxidize so readily that the digest is clear throughout the period of heating. A micro burner

² Ordinary commercial $K_2S_2O_8$ contains much ammonia. It may be purified as follows. The persulfate is dissolved in 10-fold its weight of water previously warmed to 60–70°. The solution is made strongly alkaline to litmus by addition of 40 per cent NaOH, is transferred to a vacuum distilling apparatus, and distilled under a pressure of less than 40 mm. When about one-third of the solution has been distilled off, it is replaced by an equal volume of water at 60–70°, more alkali is added if necessary to restore alkalinity to the solution, and the distillation is continued until the distillate tested by Nessler's reagent is ammonia-free. The addition of warm water and alkali may have to be repeated again before all the ammonia is removed. When ammonia has been completely removed, the persulfate solution is poured into a beaker and let stand overnight, preferably in an ice box, to crystallize. The crystals are washed with cold water on a Buchner funnel, and dried over sulfuric acid in a vacuum desiccator. Persulfate prepared this way can be obtained from Merck and Co.

is used, and during the final heating somewhat less heat is used than in the ordinary micro-Kjeldahl-Gunning digestion. The tip of the burner should just touch the bottom of the test-tube. An unnecessary intensity or prolongation of the heating may cause bumping, or corrosion of the test-tube by the phosphoric acid in the digestion mixture.

Occasionally a substance unusually difficult to digest (e.g. a lipid) may fail to clear in 15 minutes. In such a case the digest is cooled somewhat, and 0.2 to 0.3 gm. more of potassium persulfate with 2 or 3 drops of water is added.

Neutralization of Digest and Transfer to Gas Apparatus.—The digest is cooled, 3 cc. of water are poured upon it, and a drop of 1 per cent alizarin sulfonate is added. From a pipette 40 per cent sodium hydroxide is then added, a drop at a time, with occasional cooling of the tube in a cold water stream. The heat of the reaction causes solution of the solidified melt in the bottom of the tube. Addition of alkali is continued until the mixture becomes alkaline to the alizarin. 10 per cent sulfuric acid is then dropped in until the color just changes back to acid, in order to prevent loss of ammonia during subsequent deaeration.

The solution, cooled to room temperature, is poured into the cup of the 50 cc. Van Slyke-Neill blood gas apparatus, and the volume is noted. The solution is then drawn down into the chamber. Into a graduated pipette is drawn enough water to make the total volume of the solution up to 10 or 11 cc., and this water is used in three portions to rinse into the gas apparatus the drops of solution left in the test-tube.

Gasometric Determination of Ammonia Nitrogen.—The cock of the gas chamber is sealed with a drop of mercury, the chamber is evacuated, and is shaken 2 minutes to extract the air from the solution. The extracted air bubble is ejected.³ The extraction is repeated,

³ The most satisfactory technique for ejecting the gas bubble without any solution is to bring the pressure in the chamber to atmospheric by connecting the chamber with the leveling bulb and holding the latter at such a height that the mercury surfaces in chamber and bulb are on the same level. The cock connecting with the leveling bulb is then closed, and the cock at the top of the chamber is opened wide. The contents of the chamber remain quiet as the pressure was

yielding usually a very small second air-bubble, which also is ejected without permitting more than a drop of the solution to follow it up into the cup of the apparatus.

The hypobromite solution is whirled for a few seconds about the walls of its flask to permit escape of any oxygen gas that has formed in it, and 1.5 cc. are transferred to the cup of the gas apparatus. 1 cc. is run down into the chamber. The remaining 0.5 cc. is removed from the cup, and the cock is sealed with mercury. The chamber is then evacuated and shaken 2 or 3 minutes.

The gas volume is reduced to 2 cc. and the reading, p_1 mm., on the manometer is noted, together with the temperature.

The gas is then ejected from the chamber,³ the meniscus of the solution is lowered again to the 2 cc. mark, and p_2 is read on the manometer.

When sufficient material for duplicate analyses is available, one may take an amount of material containing 1 to 4 mg. of nitrogen, with 2 cc. of sulfuric-phosphoric acid mixture and 2 gm. of persulfate. The digestion is carried out in a tube marked at 25 cc. capacity. After neutralization the digest is diluted up to the mark, and 10 cc. aliquot portions are taken for gasometric nitrogen determinations.

Calculation.

$$\text{Mg. N} = (p_1 - p_2 - c) \times f$$

where f is the factor indicating the mg. of N corresponding to 1 mm. of pressure at the temperature noted. The factor is found in Table I.

$p_1 - p_2 - c$ = pressure of nitrogen gas from the substance analyzed.

c is the value of $p_1 - p_2$ obtained in a blank analysis of the reagents, which are digested with 1 cc. of water and otherwise treated as in the analysis described. With good reagents the blank, c , does not exceed 6 mm.

Under the conditions given, the hypobromite yields 96 per cent of

already atmospheric. Now with the leveling bulb resting in the ring above the chamber level, the cock connecting bulb and chamber is slowly opened. The fluid in the chamber rises slowly until by closing the cock the rise is stopped when the water has filled the capillary at the top of the chamber.

the theoretical amount of nitrogen gas. Consequently the factors in Table I give the weight of N_2 measured in the apparatus multiplied by 1.04.

TABLE I.
*Factors by Which Millimeters of Pressure Measured at 2 Cc. Volume Are Multiplied to Obtain Mg. of Nitrogen.**

Temperature.	Factor.
°C.	
15	0.003250
16	41
17	27
18	15
19	01
20	3192
21	80
22	68
23	56
24	45
25	36
26	24
27	16
28	3104
29	3092
30	80
31	71
32	60
33	50
34	40

* These factors include the empirical correction factor of 1.04, for the reaction with hypobromite.

In order to obtain the factors in Table I, the factors in Table III of Van Slyke and Neill marked "Factors for O_2 , CO , and N_2 , Sample = 1 cc., $S = 3.5$ cc., $a = 2.0$ cc., $i = 1.00$ " were multiplied by $\frac{28.08}{1000} \times 1.04 = 0.0292$.

Applications to Blood and Urine Analysis.

For determination of the total nitrogen of serum it is convenient to dilute 10-fold and digest 1 cc. of the solution, equivalent to 0.1 cc. of serum.

For albumin nitrogen in the filtrate obtained by Howe's method, in which 0.5 cc. of plasma is added to 15 cc. of 22 per cent Na_2SO_4 , 5 cc. of the filtrate are boiled down with 1 cc. of the sulfuric-phosphoric acid mixture till fumes appear. The mixture is cooled. 1 cc. of water and 1 gm. of potassium persulfate are added, and the digestion is continued as usual. Because of the sodium sulfate present it is well to add 5 cc. of water before neutralizing, to avoid crystallization of the salt in the tube. The final volume of fluid transferred to the gas apparatus is therefore 13 to 14 instead of 10 to 11 cc., but the results are not affected.

Non-protein nitrogen of blood is done on 10 cc. of trichloroacetic acid or Folin-Wu filtrate, which is boiled down in the tube with 1 cc. of sulfuric-phosphoric acid mixture before adding the persulfate plus a few drops of water.

Urines if of ordinary concentration are diluted 5-fold, if concentrated, 10-fold, and 1 cc. samples of the solution are digested.

EXPERIMENTAL.

Analyses of Pure Substances.

Substances readily soluble in water or dilute alcohol were weighed out in amounts of 100 or 200 mg. and diluted to 20 cc. 1 cc. portions, containing 5 or 10 mg. of substance, were then analyzed as above described; *viz.*, 1 cc. of 3:1 sulfuric-phosphoric acid mixture and 1 gm. of potassium persulfate were added, and the mixture was heated until it had cleared. The ammonia nitrogen was then determined with hypobromite.

Substances not readily soluble in water were weighed out in portions of 20 to 30 mg. into Pyrex test-tubes. The substance was washed down to the bottom of the tube with as little water as possible, 2 cc. of the 3:1 sulfuric-phosphoric acid mixture were added, and the mixture was heated till fumes began to come off. It was then cooled,

TABLE II.
Analysis of Known Substances.

Substances.	Preparation of solution for analysis.	Weight of substance analyzed.	Pressure of N_2 at 2 cc. volume.	Temperature.	Nitrogen found (with factors from Table I).	Nitrogen found.	Nitrogen calculated.	Time required for digest to clear after beginning to char.
		mg.	mm.	°C.	mg.	per cent	per cent	mins.
Ammonium sulfate, (NH_4) $_2$ SO $_4$.	0.9175 gm. to 200 cc. 2 cc. digested and diluted to 25 cc. 10 cc. for analysis.	3.670	252.4	24.0	0.794	21.63	21.22	Did not char.
			253.0	24.0	0.798	21.73		
		3.670	252.8	24.2	0.795	21.67		
			252.9	24.5	0.794	21.63		
Urea, CH_4 ON $_2$.	150 mg. to 100 cc. 2 cc. for digestion and analysis.	3.00	442.6	24.0	1.392	46.4	46.7	Did not char.
			441.3	24.0	1.387	46.2		
Glycocoll, $C_2H_5O_2N$.	100 mg. to 20 cc. 1 cc. for digestion and analysis.	5.00	299.5	22.5	0.948	18.96	18.70	Did not char.
			303.3	23.0	0.958	19.16		
			300.0	23.8	0.946	18.92		
Leucine, $C_6H_{13}O_2N$.	100 mg. to 20 cc. 1 cc. for digestion and analysis.	5.00	164.4	20.5	0.526	10.52	10.70	1
			161.8	20.5	0.516	10.32		
			164.2	21.0	0.523	10.46		
			164.3	21.0	0.523	10.46		
Glutamic acid hydrochloride, $C_6H_{13}O_4N$ HCl.	200 mg. to 20 cc. 1 cc. for digestion and analysis.	10.00	242.1	21.3	0.766	7.66	7.60	7-8
			244.8	21.0	0.778	7.78		
			246.6	21.5	0.782	7.82		

Acetanilide, C_8H_9ON .	200 mg. in 50 per cent alcohol to 20 cc. 1 cc. for digestion and analysis.	10.00	331.6 332.0	24.0 24.0	1.058 1.059	10.58 10.59	10.38	2
Hydroxy-6-ethoxy-acet- anilide, $C_{10}H_{13}O_3N$.	27.5 and 25.4 mg. digested and diluted to 25 cc. 10 cc. for analysis.	11.00 10.17	253.6 234.6	25.6 25.8	0.793 0.734	7.20 7.22	7.19	Not recorded.
Dimethoxychlorodiethyl- benzylamide, $C_{11}H_{15}O_2N_2$.	24.5 and 26.4 mg. digested and diluted to 25 cc. 10 cc. for analysis.	9.80 10.56	177.2 191.1	19.5 20.0	0.549 0.609	5.60 5.76	5.77	Not recorded.
<i>m</i> -Uramino-benzamide, $C_9H_9O_2N_2$.	23.2 and 21.6 mg. digested and diluted to 25 cc. 5 cc. for analysis.	4.64 4.32	337.6 316.0	23.3 24.0	1.064 0.984	22.92 23.01	22.95	Not recorded.

and 2 gm. of potassium persulfate with 1 cc. of water were added. The mixture was heated 7 to 9 minutes after it had cleared. (It was later found that this heating after clarification is unnecessary.)

TABLE III.
Nitrogen Determination in Urine.

Urine No.	Gasometric micro-Kjeldahl.				Macro-Kjeldahl.
	Urine taken for analysis.	Pressure of N ₂ at 2 cc. volume.	Temperature.	Nitrogen per 100 cc. urine.	Nitrogen per 100 cc. urine.
	cc.	mm.	°C.	gm.	gm.
1	0.200	261.4	22.0	0.414	0.422
		260.9	23.0	0.412	0.409
2	0.200	452.2	23.0	0.714	0.721
		451.4	23.0	0.713	0.728
3	0.200	202.4	23.0	0.320	0.321
		201.9	23.0	0.319	0.329
4	0.0667	233.9	23.5	1.105	1.144
		231.5	20.8	1.104	1.141
5	0.200	344.9	31.0	0.548	0.568
		347.3	21.5	0.552	0.569
6	0.0667	271.2	21.5	1.292	1.320
		276.3	22.0	1.317	1.312
		274.6	22.0	1.305	
7	0.100	329.7	22.5	1.043	1.060
		332.9	23.0	1.051	1.069
		331.9	23.0	1.048	

4 or 5 cc. of water were poured over the digest, which was then neutralized as usual with 40 per cent NaOH. It was finally diluted up to 25 cc. volume and 10 cc. portions were transferred to the gas apparatus for hypobromite treatment. The results are given in Table II.⁴

⁴ The preparations of the last 4 substances in the table were kindly furnished by Dr. W. A. Jacobs.

Analyses of Urines.

Urines were diluted 5-, or 10-, or 15-fold, according to their apparent concentration, and 1 cc. samples, equivalent to 0.2, 0.1, or 0.0667 cc. of urine, were analyzed by the gasometric method. As controls, 5 cc. samples of the undiluted urines were analyzed in duplicate by the usual Kjeldahl-Gunning method, each sample being digested with 20 cc. of sulfuric acid, 10 gm. of potassium sulfate, and a small crystal of copper sulfate. The results are given in Table III.

TABLE IV.
Repeated Analyses of One Specimen of Horse Serum.

No. of digest table.	Pressure at 2 cc. volume of N ₂ from 0.12 cc. serum.	Temperature.	Nitrogen in sample.	Nitrogen per 100 cc. serum.
	mm.	°C.	mg.	gm.
1	426.9	25.0	1.337	1.114
	426.5	25.0	1.336	1.113
2	426.7	25.3	1.336	1.113
	425.5	25.3	1.333	1.110
3	422.7	25.3	1.324	1.103
	428.1	25.3	1.342	1.117
4	430.2	25.0	1.349	1.123
	426.1	24.0	1.341	1.117
5	427.9	24.0	1.346	1.122
	427.5	24.0	1.345	1.121
Macro-Kjeldahl.....				1.113
				1.117
				1.105

Analyses of Serum.

In order to ascertain the degree of constancy attainable in the analysis of a complex biological mixture five pairs of duplicate analyses were performed on one sample of horse serum. The serum was diluted to 10 volumes with 0.85 per cent salt solution. For each digestion 3 cc. of serum solution, equivalent to 0.3 cc. of serum, were

mixed with 2.5 cc. of the 1:3 phosphoric-sulfuric acid mixture and 2 gm. of potassium persulfate. The solutions cleared after about 5 minutes heating. Each digest was diluted, neutralized with 40 per cent NaOH, and finally brought to 25 cc. volume. Of this, 10 cc. portions, equivalent to 0.12 cc. of serum each, were transferred to the gas apparatus for determination of the ammonia. Deviations between the two results from each solution indicate the degree of variability due to the final hypobromite nitrogen determination in the gas apparatus, while the differences between the different digests indicate the degree of error that may be introduced in the digestion process.

For the control determinations by macro-Kjeldahl 10 cc. portions of the 1:10 serum dilution were each digested with 20 cc. of sulfuric acid, 10 gm. of potassium sulfate, and a small crystal of copper sulfate. The ammonia was distilled into N/14 acid, of which is neutralized (after correction of 0.27 for blank) 11.13, 11.17, and 11.05 cc. in three determinations. The results are given in Table IV.

Of the ten gasometric determinations, nine gave between 1.333 and 1.349 mg. of nitrogen, maximum deviation being ± 0.5 per cent of the mean. The other result, 1.324 mg., deviated 1.2 per cent. The serum nitrogen content of 1.113 gm. per 100 cc. indicated by the average of the gasometric analyses agrees sufficiently well with the average of 1.108 gm. by the macro-Kjeldahl analyses to confirm the approximate correctness of the empirical 1.04 correction factor used in the calculation of the hypobromite results.

SUMMARY.

A micro-Kjeldahl method is described in which a quick digestion is obtained by the use of a mixture of sulfuric and phosphoric acids and potassium persulfate, and in which the ammonia formed is determined gasometrically. The neutralized digest is washed into the manometric gas apparatus of Van Slyke and Neill, and is there treated with hypobromite. The nitrogen gas is evolved in 2 minutes. Results are reproducible to within 1 per cent. The method obviates distillation and the use of standard solutions.

The greater part of the experimental work involved in developing the method here reported was carried out by John Plazin.

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THE SURVIVAL OF THE TUBERCLE BACILLUS IN SUSPENSION IN PHYSIOLOGICAL SALT SOLUTION.

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The length of time that tubercle bacilli will live and maintain virulence when suspended in physiological salt solution has never been finally determined. The matter is of interest to those who use saline suspensions of the organisms in producing experimental infections. Webb, Ryder, and Gilbert (1) have noted that a saline suspension of tubercle bacilli was able to produce tuberculosis in guinea pigs after it had stood for as long as 87 days. They did not test its virulence for longer periods of time.

This note is to place on record the observation that three suspensions of tubercle bacilli in physiological saline were alive and virulent for guinea pigs after standing for periods of 310, 325, and 330 days, respectively, at refrigerator temperature.

The refrigerator used fluctuates about the freezing point, the fluctuations not being recorded. The suspensions were made by lightly grinding the moist bacilli from active glycerol agar cultures in an agate mortar, adding 0.85 per cent NaCl solution slowly at first and then more rapidly to the amount of 1 cc. per mg. of bacilli. The suspensions were stored in narrow neck, round flasks of pyrex glass stoppered with cotton. Evaporation was not measured but was slight in amount.

Two of these suspensions were of the same strain of organism, Bovine 18, and the other suspension was of a human type, 7156. Two guinea pigs were used in determining the viability of each suspension, one receiving 0.1 mg. of organisms intraperitoneally, and the second 1 mg. in like manner.

The results are presented in Table I.

All of these animals showed a well marked generalized tuberculosis and there was nothing atypical in the course of the disease or in the autopsy findings.

Culture Bovine 18 has not been used recently in infecting guinea pigs and consequently it is impossible to state how much of its virulence has been lost during its prolonged suspension in salt solution. Culture H 7156 has, however, recently been so used. In a group of 29 guinea pigs each received 0.1 mg. of H 7156 subcutaneously. The average length of life for the group was 164 days, with the extreme variations at 88 and 230 days. The animal receiving 0.1 mg. of the 325 day old suspension of H 7156 intraperitoneally died in 145 days. It would seem then that the virulence has not been greatly diminished.

TABLE I.

Culture	Age of suspension	Dose	Length of life, in days, following infection
	<i>days</i>	<i>mg.</i>	
18	310	0.1	93
18	310	1.0	160
18	330	0.1	98
18	330	1.0	128
7156	325	0.1	145
7156	325	1.0	108

SUMMARY.

Three suspensions of tubercle bacilli in physiological salt solution were still virulent for guinea pigs after 310, 325, and 330 days at refrigerator temperature. One of these cultures on which recent tests had been made had lost very little, if any, of its virulence for guinea pigs in 325 days.

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THE BEHAVIOR IN VIVO OF CERTAIN RELATIVELY PURE ANTIGENS.

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The fact that certain proteins, such as those of blood serum, when introduced into the body will persist in the circulation for considerable periods is well known. It is also true that in certain individuals precipitin will appear in the blood before the antigen has disappeared. Thus the paradox of antigen and antibody occurring in the blood at the same time is frequently encountered. Several explanations of this phenomenon have been offered. Some have favored the view that an excess of colloid will inhibit the combination of antibody and antigen. Others have pointed out that such substances as egg white and serum are composed of several proteins and that the precipitin which appears in the blood may really be due to a single antigen which has been exhausted during the production of the antibody. The resulting antibody would react with a portion of the complex antigen and the remaining proteins would still be capable of reacting with the complex precipitin.

During the last few years the view that the paradox is explicable on the basis of multiple antigens has come into considerable prominence although in the main relatively few experiments have been reported to support this view. Those of Bayne-Jones¹ cannot be said to do so. He was able to show that rabbits immunized with crystallized egg albumen, or edestin, until their serum contained precipitin in considerable concentration would on reinjection of the specific antigen contain both antigen and antibody for a period of 48 hours. He further brought out the fact that *in vitro* much the same phenomenon was observed when antigen and antibody were mixed and the resulting precipitin centrifuged out. The supernatant fluid contained both antibody and antigen. Previously, however, R. Weil's² experiment indicated that this phenomenon held true only when multiple

¹ Bayne-Jones, S., *J. Exp. Med.*, 1917, xxv, 837.

² Weil, R., *J. Immunol.*, 1916, i, 19.

antigens were employed. Thus when horse serum or egg white was mixed with its respective antiserum and the precipitate removed by centrifugation, the supernatant liquid contained both antigen and antibody. With crystalline egg albumen the results were not the same. In these experiments he showed that both antigen and antibody never occurred together in the supernatant liquid. The fact that the presence of a third colloid failed to interfere with the interaction between antigen and antibody was demonstrated in his experiments.

The work of Opie³ follows more closely the phenomena subsequent to the therapeutic administration of horse serum. From his first series of experiments it is evident that after injection of horse serum into the blood stream of rabbits, precipitin for horse serum appears in the blood before the horse serum proteins entirely disappear. This he attributes to the composite antigenic nature of the horse serum, and suggests that certain of the contained proteins give rise to antibodies much more rapidly than certain others. When similar experiments were performed with a relatively pure crystallized egg albumen the findings were different. It is clear from Opie's findings that when crystallized egg albumen is employed as antigen the corresponding antibody and uncombined egg albumen do not appear in the blood together.

Ascoli⁴ had called attention to the fact that after the injection of egg white it appeared in the urine. Opie⁵ also found that crystallized egg albumen appeared in the urine subsequent to injection. Therefore, if such be the case, the injection of crystallized egg albumen hardly affords a parallel to the administration of foreign blood proteins. It is well known that the administration of large amounts of horse serum for therapeutic purposes does not result in the appearance of the foreign serum proteins in the urine provided the kidney is intact. The writer has on several occasions injected 5 or 6 cc. of cow serum into the peritoneal cavity of normal rabbits but in no instance has it been possible to detect it in the urine, although its presence in the rabbit's blood serum could readily be determined.

It seemed necessary to study the behavior of crystallized egg albumen after its injection to determine whether its rapid elimination might not afford an explanation for the fact that antibody failed to appear while the antigen was still present. The experiments indicate that the behavior of egg albumen is in certain respects different from that of foreign blood proteins, so that in the second series of observations casein, an antigen which on the whole affords an analogy to the blood proteins in its general behavior, was used.

³ Opie, E. L., *J. Immunol.*, 1923, viii, 55.

⁴ Ascoli, M., *Munch. med. Woch.*, 1902, xlix, 398.

⁵ Opie, E. L., *J. Exp. Med.*, 1924, xxxix, 659.

EXPERIMENTAL.

Rabbits, 2,500 gm. in weight, were immunized with various antigens and after sera of sufficient titer were obtained they were bled and the serum stored in the refrigerator. The specific precipitin was used to test the blood serum and urine for the presence of antigen. The test rabbits were of about the same age and size. The injections were made intravenous or intraperitoneal and the animals bled at frequent intervals from the ear vein. The blood was collected in sterile test-tubes and permitted to clot in a thin layer, in the incubator, and when sufficient serum had oozed out it was collected and centrifuged. In this way a clear serum was available for the tests within 2 or 3 hours.

The naturally voided urine was collected in clean bottles. It was filtered twice through filter paper.

TABLE I.

The Results of the Tests for the Presence of Antigen in the Blood Serum of Rabbit 1 Injected Intravenously with Crystallized Egg Albumen.

Time after last injection that sample was taken	Amount of serum tested				
	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.	0.05 cc.
18 hrs.	—*	—	—	—	—
42 "	—	—	—	—	—
3 days	—	—	—	—	—
4 "	—	—	—	—	—

* Since crystallized egg albumen reacts powerfully with its antibody, the intensity of the reaction has been recorded as follows: + + +, a heavy precipitate; + +, a moderate precipitate; +, slight but definite precipitation; ±, turbidity with a trace of precipitate.

The crystallized egg albumen was prepared by precipitation with ammonium sulfate after the method of Hopkins and Pinkus.⁶ It was recrystallized four times. For the casein I am indebted to Dr. J. H. Northrop of The Rockefeller Institute. It was prepared by his method.⁷

Experiment 1.—Rabbit 1. Weight, 2,245 gm. On Nov. 2, 1925, at 9.45 a.m., received 3 cc. of a 5 per cent solution of crystallized egg albumen intravenously. The animal urinated at 4.10 p.m. At 4.45 p.m., 2.5 cc. of the egg albumen was again injected into the ear vein. A blood sample was taken on Nov. 3, at 10 a.m. Blood for the tests was drawn daily and the urine was collected during the next

⁶ Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898-99, xxiii, 130.

⁷ Northrop, J. H., *J. Gen. Physiol.*, 1922-23, v, 749.

few days. The results of the tests of blood for both egg albumen and antibody are given in Tables I and II, and those of the urine in Table III.

TABLE II.

The Development of Antibody in the Blood of Rabbit 1 Subsequent to Intravenous Injection of Crystallized Egg Albumen.

Time after injection tests were made	Dilutions of antigen					
	1:40	1:80	1:160	1:320	1:640	1:1,280
<i>days</i>						
1	—	—	—	—	—	—
2	—	—	—	—	—	—
3	—	—	—	—	—	—
4	—	—	—	—	—	—
6	—	—	—	—	—	—
8	—	—	—	—	—	—
9	—	—	—	—	—	—
10	±	±	±	±	±	±
11	+	+	+	+	+	+

TABLE III.

The Appearance of Crystallized Egg Albumen in the Urine of Rabbit 1 Injected Intravenously with Crystallized Egg Albumen.

Urine voided	Dilutions of urine tested									
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
6½ hrs. after 1st injection.....	—	+	++	+++	+++	+++	++	+	+	—
During night following injection (from 2 to 16 hrs. after 2nd injection).....	—	—	+	++	+++	+++	+++	+	+	—
During 2nd night following injection.	+	±	—	—	—	—	—	—	—	—
46 hrs. after 2nd injection.....	—	—	—	—	—	—	—	—	—	—
During 3rd night following injection.	—	—	—	—	—	—	—	—	—	—

It becomes apparent that Opie's contention that crystalline egg albumen and its respective precipitin are not demonstrable together in the blood is well founded. From the results obtained in this experiment there may be some doubt that the crystallized egg

albumen precipitin was really active when used to test the rabbit's serum. It, however, was capable of reacting in the animal's urine. However, the contention that crystalline egg albumen differs in its behavior from the blood proteins of a foreign species when introduced into the body is true. From the tables it is clear that the antigen rapidly disappears from the circulation, since it could not be detected 18 hours following injection. That it rapidly passes through the kidney and in relatively large quantities is also true, since it was present in high dilutions of urine (1/640 cc.) as early as 6½ hours after its injection. Its elimination is rapid, since it only ap-

TABLE IV.

Examination of the Blood Serum of Rabbit 2 for Egg Albumen at Various Intervals after Its Injection.

Time after first injection	Dilutions of serum tested (in cc.)*							
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160
<i>hrs.</i>								
1	±	+	++	++	+	+	±	-
2	+	++	+	+	+	-	-	-
3	++	+	+	+	±	-	-	-
4	+	+	-	-	-	-	-	-
6	+	±	-	-	-	-	-	-
7	±	±	-	-	-	-	-	-
19†	-	-	-	-	-	-	-	-
43†	-	-	-	-	-	-	-	-

* In the tests of the 2, 3, 4, and 6 hour samples, 0.7, 0.75, 0.65, and 0.8 cc., respectively, were used instead of 1 cc. as indicated.

† After 2nd injection.

peared in the urine approximately 30 hours after intravenous administration. After this time it could no longer be detected. Definite antibody appeared in the blood on the 10th day following injection.

Since in the first experiment the antigen had not been detected in the circulation, it might be said that the precipitin was unable to react in the presence of an excess of colloids. To meet this objection, and as a general confirmation of the first observation, the following experiment was performed.

few days. The results of the tests of blood for both egg albumen and antibody are given in Tables I and II, and those of the urine in Table III.

TABLE II.

The Development of Antibody in the Blood of Rabbit 1 Subsequent to Intravenous Injection of Crystallized Egg Albumen.

Time after injection tests were made	Dilutions of antigen					
	1:40	1:80	1:160	1:320	1:640	1:1,280
<i>days</i>						
1	—	—	—	—	—	—
2	—	—	—	—	—	—
3	—	—	—	—	—	—
4	—	—	—	—	—	—
6	—	—	—	—	—	—
8	—	—	—	—	—	—
9	—	—	—	—	—	—
10	±	±	±	±	±	±
11	+	+	+	+	+	+

TABLE III.

The Appearance of Crystallized Egg Albumen in the Urine of Rabbit 1 Injected Intravenously with Crystallized Egg Albumen.

Urine voided	Dilutions of urine tested										
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
6½ hrs. after 1st injection.....	—	+	++	+++	+++	+++	++	+	+	±	—
During night following injection (from 2 to 16 hrs. after 2nd injection).....	—	—	+	++	+++	+++	+++	+	+	±	—
During 2nd night following injection.	+	±	—	—	—	—	—	—	—	—	—
46 hrs. after 2nd injection.....	—	—	—	—	—	—	—	—	—	—	—
During 3rd night following injection.	—	—	—	—	—	—	—	—	—	—	—

It becomes apparent that Opie's contention that crystalline egg albumen and its respective precipitin are not demonstrable together in the blood is well founded. From the results obtained in this experiment there may be some doubt that the crystallized egg

albumen precipitin was really active when used to test the rabbit's serum. It, however, was capable of reacting in the animal's urine. However, the contention that crystalline egg albumen differs in its behavior from the blood proteins of a foreign species when introduced into the body is true. From the tables it is clear that the antigen rapidly disappears from the circulation, since it could not be detected 18 hours following injection. That it rapidly passes through the kidney and in relatively large quantities is also true, since it was present in high dilutions of urine (1/640 cc.) as early as 6½ hours after its injection. Its elimination is rapid, since it only ap-

TABLE IV.

Examination of the Blood Serum of Rabbit 2 for Egg Albumen at Various Intervals after Its Injection.

Time after first injection	Dilutions of serum tested (in cc.)*							
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160
<i>hrs.</i>								
1	±	+	++	++	+	+	±	—
2	+	++	+	+	+	—	—	—
3	++	+	+	+	±	—	—	—
4	+	+	—	—	—	—	—	—
6	+	±	—	—	—	—	—	—
7	±	±	—	—	—	—	—	—
19†	—	—	—	—	—	—	—	—
43†	—	—	—	—	—	—	—	—

* In the tests of the 2, 3, 4, and 6 hour samples, 0.7, 0.75, 0.65, and 0.8 cc., respectively, were used instead of 1 cc. as indicated.

† After 2nd injection.

peared in the urine approximately 30 hours after intravenous administration. After this time it could no longer be detected. Definite antibody appeared in the blood on the 10th day following injection.

Since in the first experiment the antigen had not been detected in the circulation, it might be said that the precipitin was unable to react in the presence of an excess of colloids. To meet this objection, and as a general confirmation of the first observation, the following experiment was performed.

Experiment 2.—*Rabbit 2*, weighing 2,405 gm., was injected intravenously, at 9.30 a.m. with 3 cc. of 5 per cent crystalline egg albumen. An hour later it was bled and at intervals throughout the day. Urine was passed 5½ hours after the first injection. At 4.45 p.m., 3 cc. of the albumen solution was injected into the ear vein. As in the previous experiment, the blood was regularly tested for egg albumen and its antibody. The urine was also tested for antigen. Inasmuch as the experiment added little to the development of precipitin, the details will not be given. It is sufficient to state that antibody appeared in the blood on the 7th day. The results of the tests of the blood serum and urine for egg albumen are recorded in Tables IV and V.

TABLE V.

Examination of the Urine of Rabbit 2 for Egg Albumen after Its Intravenous Injection.

Time urine was voided	Dilutions of urine tested							
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160
5½ hrs. after 1st injection.....	—	—	±	++	++	++	+	+
During night following 2nd injection (from 2 to 16 hrs. after 2nd injection).....	±	±	+	+	++	++	+	±
18 hrs. after 2nd injection.....	±	±	±	±	±	—	—	—
During 2nd night following 2nd injection.....	—	—	—	—	—	—	—	—
63 hrs. after 2nd injection.....	—	—	—	—	—	—	—	—
72 " " " "	—	—						
4 days " " " "	—	—						

The findings in the second experiment fully confirm those of the first. The contention that crystallized egg albumen is rapidly eliminated from the circulation is well borne out. It can be readily detected in the blood during the first 3 hours following its administration, but by the end of the 4th hour it has decreased considerably. After 7 hours only a trace remains and after 18 hours it can no longer be detected. The precipitin used in the observations would react with as little as 1/700,000 gm. of the antigen. The passage of the albumen through the kidney compares in a rough way with the decline in the circulation. In both instances the urine contained the antigen in considerable concentration as early as 6 hours after its injection. Egg albumen could be demonstrated only for a period of 1 or at most 2 days in the urine. The specific antibody appeared in the blood in one instance in 7 and in the other in 10 days after the injection of the antigen.

Since crystallized egg albumen fails to meet the general requirements, the number of relatively pure proteins of general availability is somewhat limited. Bayne-Jones used with considerable success

TABLE VI.
The Behavior of Casein and Its Antibody in Rabbits 3 and 4.

Rabbit No.	Days after injection	Test of serum for casein in		Test of urine for casein in			Test of serum for antibody			
		1.0 cc.	0.1 cc.	1.0 cc.	0.5 cc.	0.1 cc.	Dilutions of antigen			
							1:100	1:200	1:400	1:800
3	1	+	*	—	—	—	—	—	—	—
	2	+	—	—	—	—	—	—	—	—
	3	+	±	—	—	—	—	—	—	—
	4	+	±	—	—	—	—	—	—	—
	6	+	?	—	—	—	—	—	—	—
	7	+	?	—	—	—	—	±	±	—
	8	+	?	—	—	—	+	+	+	—
	9	+	—	—	—	—	+	+	+	—
	10	+	—	—	—	—	+	+	+	—
	11	±	—	—	—	—	±	+	+	+
	13	±	—	—	—	—	±	+	+	+
	14	±	—	—	—	—	±	+	+	+
	16	—	—	—	—	—	—	±	+	+
4	1	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—
	3	+	—	—	—	—	—	—	—	—
	4	+	—	—	—	—	—	—	—	—
	5	+	—	—	—	—	—	—	—	—
	7	+	—	—	—	—	—	—	—	—
	8	+	—	—	—	—	—	—	—	—
	9	+	—	—	—	—	±	±	±	—
	10	±	—	—	—	—	±	+	+	—
	11	±	—	—	—	—	+	+	+	—
	12	±	—	—	—	—	+	+	+	—
	14	—	—	—	—	—	+	+	+	—

* + indicates a well defined turbidity; ±, a slight turbidity.

the globulin edestin. In my hands edestin proved unsatisfactory. When dissolved in sufficiently strong alkali it could not be injected with safety. If by titration one attempted to reduce the free alkali it would give a clear solution, but on the addition of small amounts

of normal rabbit serum a ring would form at the point of contact between the two liquids and after mixing precipitation would occur. Its use for this reason was discontinued.

Gay and Robertson⁸ and Wells and Osborne⁹ showed that purified casein is antigenic. By the repeated intraperitoneal injection of a 1 per cent solution of casein a precipitin was obtained which would in amounts of 0.1 or 0.2 cc. react with as little as 1/100,000 gm. of casein. As a rule the distinct line at the union of serum and casein occurred promptly. In the higher dilutions there is little actual precipitation but the mixed liquids promptly become turbid.

That casein, on the whole, meets the requirements of a relatively pure protein which will persist in the circulation for a considerable period is brought out in the following observations.

Experiment 3.—On different occasions rabbits were injected with casein. The casein was dissolved in N/20 NaOH and sterile salt solution. The solution was injected intraperitoneally in 3 doses during the day. Rabbit 3 received 0.6 gm. of casein; Rabbit 4 0.5 gm.

All the voided urine was collected and tested during the next 5 days. The blood serum from both rabbits was tested at frequent intervals for casein with the anticasein serum. As a control procedure tubes of the experimental animals' sera were incubated and refrigerated. In this way an accurate comparison of turbidity was possible. The urine was twice filtered through filter paper and tested in the same manner. Control tubes of the same sample were always incubated and refrigerated. The tests for casein precipitin were carried out in the usual manner. 0.2 cc. of the rabbit's serum was added to 1 cc. of the various dilutions of the antigen. The results are given in Table VI.

It is apparent that casein, like the blood proteins, remains in the circulation for considerable periods. Even as long as 12 and 13 days after its injection the serum responds weakly to the specific test. Like the serum proteins it fails to pass through the kidney in quantities sufficient to be detected. In both instances antibody was recognized in the blood serum while the casein was still in the circulation.

DISCUSSION AND SUMMARY.

The experiments are of interest in several respects. It is clear that crystallized egg albumen is rapidly eliminated from the circula-

⁸ Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1912, xvi, 470.

⁹ Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

tion and in the experiments cited it could no longer be detected after 18 or 19 hours. A considerable portion of it rapidly passes through the kidney in an apparently unaltered state. Evidently this passage begins almost at once and may continue for a day or two. In an experiment not reported in this paper, egg albumen appeared in naturally voided urine 2 hours following its injection into the peritoneal cavity. In the experiments reported no urine was voided until $5\frac{1}{2}$ and $6\frac{1}{2}$ hours following intravenous administration, but in each instance egg albumen was present in considerable amounts. However, sufficient egg albumen must have been utilized to produce antibody. It is hardly to be expected that such a protein, whose elimination is so rapid, could persist unaltered within the body and reappear within the circulation coincident with its antibody. The behavior of the protein cannot be ascribed to alterations which may have taken place during the process of crystallization since Ascoli showed that the proteins of egg white readily pass from the circulation into the urine. Certain observations of the writer confirm this point. The experience of Alexander, Becke, and Holmes¹⁰ who exposed sensitized guinea pigs to sprays of dilute egg white with the result that 80 per cent of the animals developed symptoms of anaphylaxis, further strengthens the contention that certain of the membranes are readily permeable for the proteins of egg.

The conditions following the injection of casein are different. There is no appreciable passage through the kidney. Casein is present within the circulation for a considerable period; it could be detected in the blood serum 12 and 13 days after its introduction into the peritoneal cavity. Antibody appeared on the 7th and 8th days, respectively, so that both antigen and antibody were present in the serum for a period of 3 or 4 days. The phenomenon of antigen and antibody occurring together might be explained on the ground that certain proteins are utilized slowly and that the antibody found in the blood, usually after the 7th day, results from the portion of antigen first utilized. During the next few days a continual supply of antibody enters the circulation and during the period there is a steady utilization of the antigenic substance; it is possible that during

¹⁰ Alexander, H. L., Becke, W. G., and Holmes, J. A., *J. Immunol.*, 1926, xi, 175.

this time there is constant union of antigen and antibody within the blood, with the slow utilization of the antigen and a slight utilization of the antibody which is made up by a slow increase from the body cells. Thus there would be a period in which considerable antigen would be present with weak antibody, succeeded by a second period when the amount of antigen would be small with well defined antibody, and finally only antibody. Certain observations tend to support such a view. Bayne-Jones injected rabbits whose serum contained precipitin from egg albumen with this substance and noted the occurrence of both antigen and antibody for a period of 48 hours. Some of his experiments *in vitro* are equally suggestive. In one instance a rabbit well immunized with egg albumen was injected intravenously with this substance. An hour later it was bled and the stored serum refrigerated for a period. During this time there was a slow spontaneous precipitation with a decline in both precipitin and antigen titer, but even after 6 days both were present. After a longer period only antigen remained. P. A. Lewis and D. Loomis¹¹ have shown that an injection of sheep red blood cells in guinea pigs results in a well defined hemolysin titer about the 9th day, followed by a definite decline, with a secondary rise in hemolysin until the peak is reached on the 20th day.

It becomes evident, then, that the reaction of the rabbit to a single injection of a relatively pure protein will depend on the character of the protein injected. When crystallized egg albumen is administered it is rapidly eliminated from the circulation. The rapid disappearance of the egg albumen from the blood stream is partly accounted for by its prompt elimination through the urine. Antibody appears in the serum from the 7th to the 10th day. Casein behaves differently. It persists in the blood for a considerable period; after the 7th or 8th day both antigen and antibody may be demonstrated in the blood. Casein cannot be detected in the urine following its injection into the body. The behavior of casein within the body affords an analogy with the conditions frequently noted after the administration of foreign serum, in both cases both antigen and antibody may be present in the circulation together.

¹¹ Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, xl, 503.

THE QUANTITY OF CHOLESTEROL IN THE BLOOD SERUM OF THE GUINEA PIG AS AN INHERITED CHARACTER; ITS RELATION TO NATURAL RESISTANCE TO TUBERCULOSIS, AND TO TUBERCULOSIS INFECTION.

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The literature offers certain suggestions that changes in the distribution of cholesterol may be a feature of the pathology of tuberculosis.

Caldwell (1) analyzing tuberculous bovine lymph gland and liver tissue found two to three times the cholesterol content normal for these tissues. The percentage increase varied with the degree of caseation. Jaffé and Levinson (10) observed a marked increase in optically active fat in the tubercles of rabbits fed cholesterol in oil solution. Thus it would seem that there may be an increase of cholesterol at tuberculous foci and that the content of this substance may be carried still higher by administration of the substance.

Bacmeister and Henes (4) found either normal or increased values for blood cholesterol in tuberculous patients who were doing well and in good condition, while low figures were obtained in the stage of cachexia. Chauffard, Laroche, and Grigaut (5) obtained similar results. Weltmann (6) found normal cholesterol figures in early tuberculosis, but low ones in advanced, the amount falling rapidly in acute cases. Henning (7) obtained practically normal plasma cholesterol values when using the non-saponification method of cholesterol determination, while with the saponification method he obtained results uniformly much lower. He considered that this indicated the presence, in the blood of tuberculous individuals, of some substance other than cholesterol which gives the cholesterol color reaction but which is sensitive to alkali. Sweany, Weathers, and McCluskey (8) failed to find such a marked difference when using these two methods although the saponification method gave slightly the lower blood cholesterol values. This difference was not more marked, however, than have been noted (9) on a comparison of the two methods in conditions other than tuberculosis. They found that blood cholesterol values were highest in tuberculous patients who were doing well and low in patients doing poorly and losing ground.

Jaffé and Levinson (10), using rabbits infected with bovine type tubercle bacilli, found that following infection the blood cholesterol began to rise from a normal of 80 and 90 mg. per cent to 115 to 130 mg. per cent. In one animal this high value was maintained, while in the other animal it dropped before death to almost the original value.

The authors cited agree that the blood cholesterol is decreased in the more advanced stages of tuberculosis. In the earlier stages normal or increased values have usually been found. This refers to human beings and to rabbits. I have found no reference to determinations of blood cholesterol on either the normal or tuberculous guinea pig.

The inbred guinea pigs as used by Wright and Lewis (2), and Lewis and Loomis (3), are being maintained. In view of the variations in natural resistance to tuberculosis existing among inbred families of guinea pigs, it seemed not impossible that these animals might show some differences referable to their cholesterol metabolism. Dr. Lewis has kindly put his stock at my disposal for determinations of the cholesterol of the blood serum.

These experiments were therefore planned to determine the influence, if any, of an infection with the tubercle bacillus of bovine type on the cholesterol content of the blood of the guinea pig; and also to determine the normal cholesterol content of the blood of the available inbred guinea pigs, with particular reference to any familial variation.

EXPERIMENTAL.

It was found that there was a large individual variation in blood cholesterol, especially pronounced with the females. Observations were therefore confined to large groups of males.

Sixteen male guinea pigs from inbred Family 35 and the same number from inbred Family 13 were bled 1 cc. from the heart, and the 16 cc. of blood thus obtained was pooled and allowed to clot in the refrigerator. The clot was later broken up and the specimens centrifuged to obtain the serum. 3 cc. quantities were used in the determinations, and Bloor's method (9) was employed throughout with the modification that the amounts of alcohol-ether extractive were doubled because of the low serum cholesterol values in guinea pig blood. By using 20 cc. of alcohol-ether extract instead of 10 cc., colors readable in the colorimeter could be obtained. The cholesterol value obtained in this way had of course to be

TABLE I.

Uninfected Animals		
Bled Feb. 4, 1926. Diet: Hay, oats, and mangels		
Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
13	59.5	58
	58.6	
	55.8	
35	64.6	65
	64.5	
	64.5	
Feb. 5, 1926, diet changed to hay and soaked oats daily, cabbage once a week		
Bled Feb. 9, 1926		
13	56	56
	57	
	57	
	55	
35	69	69
	66	
	71	
	69	
Bled Feb. 15, 1926		
13	61	60
	60	
	60	
	59	
35	67	68
	68	
	67	
Feb. 18, 1926, infected with 1/10 mg. of a culture of bovine tubercle bacilli (Bov. 14) intraperitoneally		

TABLE I—*Continued.*

Infected Animals		
Bled Feb. 23, 1926		
Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
13	58	58
	58	
35	63	64.5
	66	
Bled Mar. 1, 1926		
13	57	57
	56	
	57	
	57	
35	75	71
	73	
	68	
	68	
Bled Mar. 8, 1926		
13	60	59
	59	
	60	
	58	
35	65	66
	64	
	67	
	68	
Bled Mar. 18, 1926		
13	59	59
	59	
	60	
	59	
35	67	68
	67	
	68	
	68.5	

TABLE I—*Concluded.*

<i>Average for Total Experiment</i>		
	Family 35	Family 13
Before infection.....	67 mg. per cent	58 mg. per cent
After "	67 " " "	58 " " "

divided by two. Each specimen of serum was run in duplicate and the extractives, in turn, were run in duplicate in most cases.

The animals were bled three times at intervals of 5 or 6 days. They were then inoculated intraperitoneally with a virulent culture of the tubercle bacillus of bovine type and again bled at intervals of 7 to 10 days after the infection had been established. Animals killed accidentally while bleeding were replaced, until the time of inoculation.

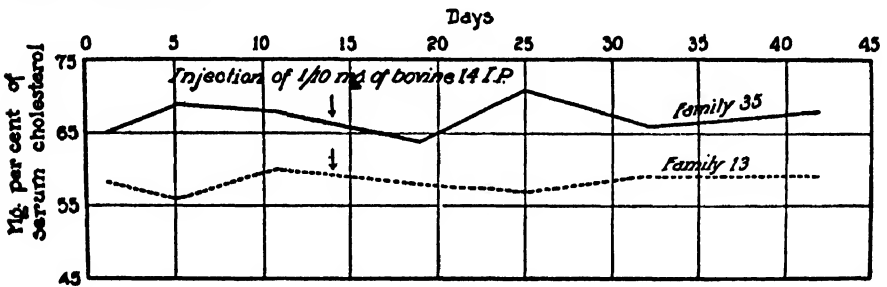


CHART 1.

The results of the serum cholesterol determinations are given in Table I, and graphically in Chart 1.

From these results it is evident that a tuberculous infection in guinea pigs produces no marked change in the serum cholesterol values. There was no rise at the onset of infection and no terminal fall was noted, although at the time of the last bleeding the animals were losing weight rapidly and had begun dying of tuberculosis. The animals in Family 35 showed uniformly higher serum cholesterol values than did the animals in Family 13. For reasons apart from present considerations, the diet was changed after the first bleeding of the period prior to infection and the diet then established was maintained throughout. The change was evidently without effect on the cholesterol content of the serum.

A further series of examinations was now conducted involving guinea

pigs from each of the four inbred families whose reactions to tuberculous infection are known. In this case a change of diet was also introduced following the first bleeding and 2 weeks before the second.

TABLE II.

Bled Mar. 30, 1926. Diet: Timothy hay, oats, and mangels		
Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
13	57	57
	57	
35	66	66
	66	
32	64	62.5
	61	
2	49	50
	51	

Apr. 1, 1926, diet changed to alfalfa hay and oats, cabbage once a week		
Bled Apr. 13, 1926		
13	55	55
	55	
35	61	65
	69	
32	66	66
	66	
2	52	52.5
	53	

Since the previous results had made it apparent that tuberculous infection produced no changes in the serum cholesterol values in guinea pigs, the determinations this time were only on normal, uninfected animals. Eight male guinea pigs of about the same weight and age from each family were used for this experiment and the blood was handled in the same way as before. The results are given in Table II.

In this series, guinea pigs of Family 35 again showed a uniformly higher serum cholesterol content than those from Family 13 and the values obtained for each family are comparable to those obtained in the first experiment. Guinea pigs of Family 32 have values very close to those for Family 35, while the figures obtained for Family 2 are lower than those found for Family 13. On the basis of serum cholesterol the order of the families from high to low is 35, 32, 13, 2.

The substitution of alfalfa for timothy hay in the diet had no apparent effect on serum cholesterol concentration.

TABLE III.

No. of guinea pig	Family	Serum cholesterol	
		Separate readings in mg. per cent	Average for families in mg. per cent
1	13	50	50
2		48	
3		50	
4		52	
5	35	63	59.6
6		58	
7		59.5	
8		58	

In order to determine how wide might be the individual variations among the animals of Families 13 and 35, serum cholesterol determinations were run on four male guinea pigs from each of the two families. 8 cc. of blood was drawn from the heart of each animal and 3 cc. of serum was used in the determination, following the same method as in the previous analyses. The results are recorded in Table III.

In this series of determinations the serum cholesterol values ran uniformly lower than in the preceding series. This may have been due to the fact that early in May, 2 weeks prior to the bleedings, the animals were put on a succulent diet consisting of freshly cut clover and alfalfa. Practically the same differences prevail between the two families although on a lower level. In the first experiment the average values obtained were, for Family 35, 67 mg. per cent, and for

Family 13, 58 mg. per cent, a difference of 9 mg. per cent. In this series the difference noted in the average of the values is 9.6 mg. per cent. It is also to be noted that the individual variation appears to be from 4 to 5 mg. per cent. Comparison with the tables of the earlier experiments shows that this is within the limits of variation shown by the separate readings on the same blood sample and indicates that the individual differences cannot, with the technic employed, be regarded as significant.

DISCUSSION.

The results obtained have interest in several directions. It is clear that the progress of tuberculosis in the guinea pig is not marked by changes in serum cholesterol. This is in suggestive contrast to the observations on the human disease and to the experiments of Jaffé and Levinson on the rabbit. The differences may depend on a different relationship due to species, but it is also possible that the changes found by previous observers, which have been changes in the cholesterol content of the whole blood in most cases, may be due to changes in the corpuscles.

Any changes in diet under controlled conditions, as in Experiments 1 and 2, were without appreciable influence on the serum cholesterol. This was to have been expected since the cholesterol and phytosterol concentrations of the interchanged foods were probably not very different and the change being from one dry food to another the water exchange was probably not affected. The lower values obtained in the third series of observations might well be due to the succulent diet being fed at the time, or to other seasonal changes.

The familial differences in serum cholesterol have been consistent and are of wide interest. The families of guinea pigs used had been strictly inbred for many generations and may be regarded as homozygous. Differences between them in other respects are pronounced and constant. They relate to such characters as color, conformation, size, and fertility, and have been fully discussed by Wright (11). The concentration of serum cholesterol appears to be a well defined character peculiar to the family, and hence, considering the nature of the animal material, one controlled by the inheritance. We are familiar

with only one other recorded instance of an inheritable character related to a particular feature of the general metabolism. Benedict (12) has described peculiarities in the purine metabolism of the Dalmatian coach hound which have a similar relationship. It is of importance that our knowledge of similar instances should be multiplied as preliminary to a more definite association of genetics with physiology and pathology.

The families of guinea pigs employed for the present work possess different levels of natural resistance against infection with the tubercle bacillus (2). Wright and Lewis were led by a comparison of results with crosses between the families to assume that the total of differences found must be due to the interrelated operation of three or more separately inherited characters. The question now arises whether the serum cholesterol content is one of the three or more factors predicated by these authors. The order of familial resistance from high to low as found by Wright and Lewis was 35, 2, 32, 13. The order based on the serum cholesterol concentration from high to low is 35, 32, 13, 2. These relations make it plain that the cholesterol concentration cannot be the only factor in determining the resistance to tuberculosis, nor can it be the controlling factor.

SUMMARY.

1. Infection with tubercle bacilli of bovine type produced no alteration in the serum cholesterol content in guinea pigs.

2. Certain controlled variations in diet produced no changes in the serum cholesterol content in guinea pigs.

3. Inbred families of guinea pigs known to manifest differing resistances to tuberculosis gave differing serum cholesterol values, but no direct relationship between the two sets of phenomena could be established.

4. It seems probable that in the guinea pig the cholesterol content of the blood serum is influenced by inherited factors.

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